

1 **TITLE:**

2 A rapid intrinsic heart rate resetting response with thermal acclimation in rainbow trout,
3 *Oncorhynchus mykiss*

4 **RUNNING TITLE**

5 Pacemaker rate resetting in rainbow trout

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15 **KEYWORDS**

16 Temperature acclimation; heart; intrinsic heart rate; pacemaker; rainbow trout; HCN proteins

17 SUMMARY STATEMENT

18 In response to warm temperature, rainbow trout rapidly reset intrinsic heart rate resetting within 1 h
19 without associated changes in mRNA expression of related cardiac proteins.

20 ABSTRACT

21 We examined cardiac pacemaker rate resetting in rainbow trout following a reciprocal temperature
22 transfer. In a first experiment performed in winter, 4°C-acclimated fish transferred to 12°C reset
23 intrinsic heart rate after 1 h (56.8 ± 1.2 to 50.8 ± 1.5 bpm) and 12°C acclimated fish transferred to 4°C
24 reset intrinsic heart rate after 8 h (33.4 ± 0.7 to 37.7 ± 1.2 bpm). However, in a replicate experiment,
25 performed on a different brood year in the summer, intrinsic heart rate was not reset, even after 10
26 weeks. Using this serendipitous opportunity, we examined mRNA expression of a suite of proteins in
27 sinoatrial node (SAN), atrial and ventricle tissue after 1 h and longer than 3 weeks to assess changes
28 associated with pacemaker rate resetting in both experimental groups. In addition to the changes in
29 mRNA expression associated with thermal acclimation in contractile cardiac chambers, we observed
30 downregulation of *NKA $\alpha 1c$* in the atrium and ventricle, and upregulation of *HCN1* in the ventricle
31 after > 3 weeks of warm acclimation associated with pacemaker resetting. However, we found no
32 SAN mRNA expression changes at any time during warm acclimation, including the first hour, unique
33 to the fish with pacemaker rate resetting. Thus, despite identifying changes in mRNA expression of
34 contractile cardiac tissues, there was absence of changes in mRNA expression directly involved with
35 the initial, rapid pacemaker rate resetting with warm acclimation. Importantly, pacemaker rate
36 resetting with thermal acclimation does not always occur in rainbow trout.

37 INTRODUCTION

38 In ectothermic organisms such as fishes, acute increases in temperature result in an increase in
39 metabolic oxygen demand due to a thermodynamic response. Nevertheless, over time, many fish
40 species can acclimate to a new temperature and improve function. Thermal acclimation, as a whole,
41 involves generalized (e.g., homeoviscous adaptation) and tissue specific changes, likely over different
42 timescales (Fangue et al., 2014; Fu et al., 2018). This study focuses on the resetting of the intrinsic
43 cardiac pacemaker rate known to occur in rainbow trout (*Oncorhynchus mykiss*) during thermal
44 acclimation.

45 Intrinsic heart rate is the rate of the heartbeat without any neural or humoral influences. These
46 neural and humoral effects are largely cholinergically and adrenergically mediated in fishes and
47 respond to temperature (Altimiras and Axelsson, 2004; Ekström et al., 2016; Gilbert et al., 2019;
48 Graham and Farrell, 1989; Jose and Taylor, 1969; Wood et al., 1979). Thus, intrinsic heart rate can be

49 measured *in vivo* by removing these influences with pharmacological agents (Ekström et al., 2016;
50 Jose and Taylor, 1969). Also, intrinsic heart rate can be measured *in vitro* by placing the heart in
51 physiological saline and physically removing it from these influences, as in the present study, because
52 the fish heart is myogenic and beats rhythmically by itself (Graham and Farrell, 1989; Hiroko et al.,
53 1985). The myogenic heartbeat is a direct result of specialised pacemaker cells found in the sinoatrial
54 node (SAN) that generate spontaneous action potentials (Farrell and Smith, 2017; Haverinen and
55 Vornanen, 2007; Newton et al., 2014; Stoyek et al., 2015; Stoyek et al., 2016). Thus, the spontaneous
56 depolarisation in SAN pacemaker cells sets intrinsic heart rate, which is then modulated by neural
57 and humoral influences to set heart rate *in vivo*.

58 Intrinsic heart rate is not necessarily fixed throughout the life of an animal. For instance, in
59 ectotherms, acute warming typically increases the rate of SAN pacemaker cell depolarisation driving
60 an increase in intrinsic heart rate (Aho and Vornanen, 2001) and thermal acclimation can reset the
61 pacemaker rate. The effects of thermal acclimation on intrinsic heart rate have been studied in a
62 wide range of ectothermic species, including in rainbow trout, goldfish, perch, guppy, medaka,
63 American bullfrogs and common and wood frogs (Bowler and Tirri, 1990; Harri and Talo, 1975;
64 Hiroko et al., 1985; Lotshaw, 1977). Specifically, warm acclimation resets the cardiac pacemaker to a
65 slower rate in rainbow trout: 4°C-acclimated rainbow acclimated to 17°C decreased intrinsic heart
66 rate from 31 bpm to 25 bpm when measured at 4°C and from 74 bpm to 61 bpm when measured at
67 14°C (Aho and Vornanen, 2001). Thus, thermal acclimation in rainbow trout resets the pacemaker
68 rate in the opposite direction to that of the acute thermal response, which may then allow for
69 further increases in heart rate, for example with acute warming or exercise, before reaching the
70 maximum heart rate at the new acclimation temperature. What is less clear concerning pacemaker
71 resetting for rainbow trout is the timescale for this thermal acclimation response. General
72 assumptions are that pacemaker resetting takes several weeks at the new temperature, as reported
73 by Aho and Vornanen (2001). Indeed, Ekström et al. (2016) concluded that pacemaker rate resetting
74 in rainbow trout must occur over longer than 6 weeks because they found no change in intrinsic
75 heart rate between 1 day and 6 weeks after rainbow trout were moved from 9°C to 16°C. However,
76 alternative explanations for this result could be that either pacemaker resetting may occur rapidly, or
77 under certain conditions it may not occur at all. In fact, the ability to reset pacemaker rate with
78 thermal acclimation is currently assumed to be a taxon-specific response (Matikainen and Vornanen,
79 1992), and may even only occur over a restricted temperature range and under certain conditions.
80 For example, crucian carp acclimated to 5°C and 15°C did not reset pacemaker rate when
81 measurements were made between 4°C and 35°C (Matikainen and Vornanen, 1992). Similarly,
82 pacemaker rate was not reset in either Atlantic cod or bald rock cod (Egginton and Campbell, 2016;
83 Lurman et al., 2012).

84 Therefore, the aims of our study were two-fold: a) to resolve the time course of pacemaker rate
85 resetting in rainbow trout; and b) to provide new mechanistic insights into intrinsic heart rate
86 resetting by measuring gene expression of a suite of proteins potentially involved in the ionic
87 currents associated with cardiac activity in the sino-atrial node [SAN], atrium and ventricle because
88 such studies are quite limited for salmonids (Aho and Vornanen, 2001; Hassinen et al., 2007;
89 Hassinen et al., 2008; Hassinen et al., 2017; Haverinen and Vornanen, 2007; Korajoki and Vornanen,
90 2009; Korajoki and Vornanen, 2012). We specifically targeted proteins central to two competing
91 models advanced for pacemaking in mammals (Lakatta and DiFrancesco, 2009): a) the calcium clock
92 hypothesis, which suggests that spontaneous depolarisation is caused by spontaneous release of
93 calcium sparklets from the sarcoplasmic reticulum via ryanodine receptors; and b) the membrane
94 clock hypothesis, which suggests that spontaneous depolarisation is caused by hyperpolarisation-
95 activated cyclic nucleotide-gated (HCN) channels resulting in a “funny” current. In doing so, and
96 unexpectedly, we found that pacemaker resetting did not occur in both our acclimation experiments
97 with rainbow trout. As a result, we used a Fluidigm microfluidics qPCR platform to evaluate mRNA
98 expression of 28 functional proteins in 3 different cardiac tissues from rainbow trout acclimated to
99 either 4°C or 12°C for more than 3 weeks (4-10 weeks). Differences in mRNA expression of functional
100 genes in the SAN, atrium and ventricle were established, and differences associated with thermal
101 acclimation were identified for each tissue type. These characterizations then allowed identification
102 of functional genes with mRNA expression patterns uniquely associated with the demonstrated
103 slowing of pacemaker rate with warm acclimation in the first acclimation experiment. Thus, by
104 comparing gene expression patterns across these experiments, we were able to distinguish the
105 changes in mRNA expression potentially associated with pacemaker rate resetting responses during
106 warm acclimation from the more general cardiac responses to warm acclimation.

107 MATERIALS AND METHODS

108 Study design

109 The time-course of pacemaker rate resetting was followed during both cold-acclimation (4°C) and
110 warm-acclimation (12°C). We measured intrinsic heart rate after as little as 1 h, and as long as 10
111 weeks after transferring rainbow trout either from 4°C to 12°C, or from 12°C to 4°C (Table 1). We
112 predicted that pacemaker rate resetting would take longer to occur with cold acclimation than with
113 warm acclimation due to basic thermodynamics. However, mRNA expression in SAN, atrial and
114 ventricle tissues was only examined during warm acclimation. The experiments were performed in
115 accordance with the Animal Care Guidelines at the University of British Columbia (UBC) (Permit
116 Number - A15-0035).

117 Fish supply, temperature treatments and sampling 118 times

119 Rainbow trout were obtained from the Sun Valley Trout Hatchery, where they had been reared in
120 outdoor, flow-through raceways. Two replicate experiments were performed, one over winter from
121 November through December of 2016, and a second on a subsequent brood year of trout from the
122 same source over early summer from March through June of 2017.

123 Overall experimental design was the same for the two experiments. First, fish were pre-acclimated
124 to either 4°C ($\pm 1^\circ\text{C}$) or 12°C ($\pm 1^\circ\text{C}$) in recirculating tanks inside an environmental chamber for 3-12
125 weeks. Daily replacement with temperature-adjusted freshwater ensured good water quality,
126 ammonia and oxygen levels (monitored daily). Fish were fed a daily maintenance diet of BioTrout 4.0
127 mm (Skretting, Vancouver) and held at a 12:12 h photoperiod. A subset of fish was sampled directly
128 from the pre-experimental acclimation tanks (0 h; > 3 weeks; 10-12 weeks). Sub-groups of fish were
129 then placed into individual, 19 L mesh-covered buckets and moved into a new experimental tank at
130 the opposite temperature, and were sampled 1 h, 8 h, and 24 h at the new acclimation temperature
131 (*Fig. S1-A*). This facilitated taking rapid samples during the initial stages following transfer. As control
132 for fish handling, a group of 4°C-acclimated fish were placed in buckets and returned to the 4°C
133 experimental tank and sampled after 1 h.

134 Most of the fish used in experiment 1 were obtained in May 2016 (43 fish) and were held in outdoor
135 flow-through tanks at UBC until October 2016, after which they were moved into temperature-
136 controlled tanks for the pre-acclimation period. In November of 2016, additional fish of the same
137 brood year (27 fish) were obtained from the same source added to the pre-acclimation tanks to allow
138 increased sample sizes. Molecular samples were taken over 2 days in December from fish acclimated
139 for 1 h to 12°C, for 1 h at 4°C (a fish handling control group) and for > 3 weeks at both 4°C and 12°C
140 (7 weeks and 4 weeks, respectively). Heart rate measurements were taken between late November
141 and late December 2016. The fish used in the experiment had body mass = 337 ± 9 g (mean \pm s.e.m.);
142 $n = 70$.

143 Fish for experiment 2 were placed directly into temperature-controlled pre-acclimation tanks at UBC
144 in March 2017. The experiment was conducted as described above. Molecular samples were taken
145 during one day in May from fish acclimated for 10 weeks to either 4°C or 12°C (referred to as > 3
146 weeks acclimation). Heart rate measurements were taken between early April and early June 2017.
147 The fish used in this experiment had body mass = 258 ± 8 g; $n = 55$. Consequently, the fish used in
148 these two experiments were of the same strain, a similar size and received the same

149 experimental treatments, but they were from different brood years and were acclimated at different
150 times of the year.

151 Intrinsic heart rate measurements

152 Fish were individually euthanized with a blunt blow to the head, followed by destruction of the brain
153 and spinal cord. The heart, including much of the sinus venous to ensure an intact SAN, was quickly
154 excised (<2 min) and was placed (with the ventricle laterally cut to aid saline perfusion) in an
155 oxygenated (100% oxygen) saline bath held at the appropriate acclimation temperature (either $4 \pm$
156 1°C or $12 \pm 1^\circ\text{C}$) maintained by a circulating water bath and recirculating chillers. The temperature of
157 the physiological saline was monitored directly and contained: 140 mM NaCl, 2.8 mM KCl, 1.2 mM
158 MgSO_4 , 1.8 mM CaCl_2 , 1.2 mM NaH_2PO_4 , 3.9 mM TES free acid, 6.1 mM TES Na salt and 10 mM
159 glucose with a pH of 7.8 at 10°C . The electrocardiogram (ECG) signal was recorded using two custom-
160 made stainless steel electrodes, one placed next to the SAN the other away from the heart (*Fig. S1-*
161 *B*). The ECG signal was amplified (100-1000x) and filtered (high pass: 0.3 Hz, low pass: 0.1 kHz) with a
162 Grass P55 AC amplifier (Astro-Med Inc). Data acquisition used a PowerLab ML870 and ECG signals
163 were digitally filtered (60 Hz line filter, 0-5 Hz high pass, 45 Hz low pass) and analysed using
164 automated beat detection in LabChart 7 Pro software (AD instruments). After a stabilization period of
165 30 min in the saline bath, the stable intrinsic heart rate (bpm) was recorded as an average from
166 consecutive ECG waveforms (> 1 min) using automated beat detection. Intrinsic heart rates are
167 presented as mean \pm s.e.m. for n individuals. Fish were discarded if either the saline temperature
168 occasionally drifted outside the experimental range after 30 min, or heart rate occasionally became
169 arrhythmic or unstable. One-way ANOVAs with a post hoc Tukey test and student t-tests were
170 conducted using Sigmaplot (ver. 13.0; Systat Software) and differences were considered significant
171 when $P < 0.05$.

172 Quantitative reverse transcriptase (qRT-) PCR

173 CARDIAC TISSUE SAMPLING

174 Hearts from fish undergoing temperature acclimation were similarly removed into oxygenated saline
175 to allow dissection of the ventricle (335 ± 20 mg), atrium (58 ± 4 mg) and SAN-regions (34 ± 2 mg)
176 (*Fig. S1-C*). Each of these regions was placed into individual Eppendorf tubes for flash freezing with
177 liquid nitrogen. The SAN region can include the sino-atrial canal (Haverinen and Vornanen, 2007;
178 Newton et al., 2014; Stoyek et al., 2015; Stoyek et al., 2016), and so the considerable sample of the
179 sinus venosus tissue included a minimal amount of atrial tissue to ensure inclusion of pacemaker
180 cells. Tissues were stored at -80°C until analysed.

181 Cardiac genes for qRT-PCR targeted those potentially implicated in pacemaker function and
182 temperature acclimation (Table A1). All genes had been previously sequenced in rainbow trout
183 (D'Souza et al., 2014; Hassinen et al., 2008; Korajoki and Vornanen, 2009; Korajoki and Vornanen,
184 2012) with the exception of the HCN genes, which have not been previously sequenced. Primer
185 sequences (Table A1) were designed using Primer Express 3 (Life Technologies, Carlsbad, California,
186 USA) with melting temperatures of 58-60°C (default settings). Specificity of each primer set was
187 tested by cloning and sequencing. Each primer set was run individually on a CFX96 Touch Real-Time
188 PCR Detection System (Bio-Rad, Hercules, California, United States) with SYBR™ Green PCR Master
189 Mix (Applied Biosystems, Foster City, California, United States) and a mixed cardiac tissue sample.
190 Cycling conditions were: 95°C for 10 min followed by 40 repeats of 95°C for 15 s and 55°C for 1 min.
191 Products were cloned using TOPO™ TA Cloning™ Kit for Subcloning (Invitrogen, Carlsbad, California,
192 United States), as per manufacturer's protocol. One Shot™ TOP10 chemically competent *E. coli* cells
193 (Invitrogen, Carlsbad, California, United States) were used for most assays; however, HCN isoforms
194 were tested using Shot™ TOP10F' *E. coli* cells (Invitrogen, Carlsbad, California, United States) as these
195 produced a higher yield. Plasmid extraction was performed using GeneJET Plasmid Miniprep Kit
196 (Thermo Scientific, Waltham, Massachusetts, United States) and the resulting product was
197 sequenced by either the NAPS core facility at UBC (Vancouver, BC, Canada) or MacroGen USA
198 (Rockville, MD). Primers tested produced at least 9-10 clones of the expected sequence, and if not,
199 the primers were discarded. In most cases and apart from isoforms of HCN, we did not try to
200 distinguish between paralogues, unless previously identified in other work. When primer sequences
201 were obtained from previous studies (Table A1), the products of these primers were not cloned and
202 sequenced.

203 RNA EXTRACTION

204 RNA was extracted according to the manufacturer's protocol from all tissue samples using
205 MagMAX™-96 for Microarrays Total RNA Isolation Kits (Ambion, Austin, Texas, United States). Briefly,
206 a tissue sample was weighed, and if necessary divided into a tissue sections weighing <90 mg. A
207 tissue section was then placed into 200 µL of TRI reagent® (Sigma-Aldrich, St Louis, Missouri, United
208 States) and briefly homogenised using a pellet pestle. More TRI reagent® was added to give a final
209 TRI reagent® volume of 15 µL per 1 mg of tissue. 0.2 µL of external standard mRNA (500 pg µL⁻¹) per
210 mg of tissue was then added to the tube with □10 @ 1.0 mm ceria-stabilized zirconium oxide beads
211 (Next Advance, Averill Park, New York, United States) in each tube. The tissue samples were fully
212 homogenised with Bullet Blender24 (Next Advance, Averill Park, New York, United States) before
213 adding 0.1 mL of 1-bromo-3-chloropropane per 1 mL of Tri reagent® to each tube and centrifuging at
214 12,000 g for 10 min. The aqueous layer was removed and frozen at -80°C for storage. The aqueous
215 layer for tissues weighing >90 mg, which had been processed in sections up till this point, were

216 recombined with volumes relative to section weight, to make up 150 μL [(section weight/ sample
217 weight) x 150 μL]. When all samples had been processed to this point, the aqueous layers were
218 defrosted and placed in 96 well plates. RNA extraction then proceeded as in the 'spin' protocol using
219 a Biomek FXP (Beckman Coulter, Brea, California, United States) automated liquid handling
220 instrument. Purity was assessed using 260/280 ratios (1.97-2.45) and yield was calculated using 260
221 nm absorbance. The RNA concentration was then normalised using the BioMek FXP (Beckman
222 Coulter, Brea, California, United States) automated liquid handling instrument to 62.5 ng μL^{-1} .

223 FLUIDIGM QRT-PCR

224 qRT-PCR was run using the Fluidigm BioMarkTM microfluidics platform (Fluidigm, South San Francisco,
225 California, United States) with Evagreen[®] assays as fully described in Jeffries et al. (2014) for 33
226 target genes and 8 reference genes (*Table 2.1*). Briefly, total RNA was used to synthesize cDNA with
227 SuperScriptTM VILOTM MasterMix (Invitrogen, Carlsbad, California, United States) as per the
228 manufacturer's protocol. cDNA was pre-amplified in a specific target amplification (STA) step using
229 all the primer pairs (*Table A1*) and TaqManTM Preamp Master Mix (Life Technologies, Carlsbad,
230 California, USA). The BioMark protocol for pre-amplification was then followed (1.25 μL of cDNA,
231 1.25 μL of 200 nM pooled primer mix and 2.5 μL of Master Mix). Any primers that were
232 unincorporated were then removed using ExoSAP-ITTM High-Throughput PCR Product Clean Up (MJS
233 BioLynx Inc, Brockville, Ontario, Canada) (2 μL of ExoSap-IT per 5 μL of post-PCR reaction volume),
234 followed by 1:5 dilution in DNA Suspension Buffer (TEKnova, Hollister, California, United States).

235 qRT-PCR was run with 96 by 96 dynamic arrays using the Biomark HDTM. A sample premix (2.5 μL 2x
236 SsoFastTM EvaGreen[®] Supermix with low ROX (Biotium, Fremont, California, United States), 0.25 μL
237 20x DNA Binding Dye Sample Loading Reagent (Fluidigm, South San Francisco, California, United
238 States) and 2.25 μL of the diluted pre-amplified product) and an assay premix (2.5 μL of 2x Assay
239 Loading Reagent (Fluidigm, South San Francisco, California, United States), 2.25 μL 1x DNA
240 Suspension Buffer (TEKnova, Hollister, California, United States) and 50 μM each of mixed forward
241 and reverse primers) were made. These were loaded onto the 96.96 dynamic array and mixed using
242 an IFC controller HX (Fluidigm, South San Francisco, California, United States). qRT-PCR was then run
243 using the recommended protocol for Evagreen, GE Fast 96 x 96 PCR+Melt v2 protocol with Biomark
244 HDTM (A thermal mixing protocol of 70°C for 40 min and 60°C for 30 s, then a hot start protocol of
245 95°C for 60 s, followed by 30 qRT-PCR cycles of 96°C for 5 s and 60°C for 20 s. A melting protocol of
246 60°C for 3 s followed using a 1°C increase every 3 s up to 95°C).

247 QUALITY CONTROL

248 The same number of samples (n=7) were used for each experimental group to ensure a balanced
249 statistical design with ANOVA analyses. mRNA extraction and qPCR was performed on all original 7-
250 10 tissue samples per group and sample sizes were reduced by first excluding any individual where
251 the SAN, atrial or ventricle sample was unavailable, and then by excluding the last samples that were
252 homogenised.

253 While using the Fluidigm approach offers the advantage of running many assays simultaneously, it
254 can be difficult to optimize the conditions so that all assays perform well with differing mRNA
255 expression levels. As the aim of this study was only to gain a broad-scale picture, we did not
256 optimise and rerun assays that failed quality criteria, and instead we chose to exclude data for any
257 genes that did not meet our stringent quality control filters. Regardless, we report all efficiencies, r^2
258 and experimental Ct ranges in *Table S1*.

259 Of 33 possible target genes, 28 met our quality control standards and were ultimately used for qRT-
260 PCR analysis. For the target genes that were excluded, the standard curves for α_{1A} -*adrenoceptor*, β_2 -
261 *adrenoceptor*, *HCN4b* and *NKA $\alpha 1b$* were insufficient, while for *ANP* Ct values for some samples were
262 too low for the Fluidigm system (*i.e.*, 2.5Ct). For the remaining 28 target genes, a measurement was
263 excluded from further analysis if the difference between technical replicates was greater than 1 cycle
264 (*i.e.*, *Na_v1.4* in a single SAN sample from the fish acclimated to 4°C for 1 h with a pacemaker rate
265 resetting response, and *Na_v1.6* in a single ventricle sample from fish acclimated to 4°C for >3 weeks
266 without a pacemaker rate resetting response; *Table S1*).

267 We utilized data for two reference genes (*CCDC84* and *SEP15*) that have been used previously with
268 salmonids (*CCDC84*; Jeffries et al., 2014) and zebrafish (*SEP15*; Xu et al., 2016). Both were confirmed
269 as a suitable combination for an endogenous control using Normfinder (combined stability value of
270 0.019). Six of the reference genes tested (*18s*, *β -actin*, *EF1 α* , the external standard, *DnaJA2* and
271 *MprPL40*) were excluded using one of the following strict quality criteria: a) Ct values were outside
272 the recommended detection limit (*i.e.*, 6-25 Cts) for the Fluidigm system (*i.e.*, *18s*, *β -actin*, *EF1 α* ,
273 *DnaJA2* and *MprPL40*) (*Table S1*); b) the efficiency was $\leq 80\%$ or $\geq 120\%$ (*18s* and the external
274 standard, respectively); or c) r^2 was ≤ 0.980 (both *18s* and the external standard). Stricter quality
275 criteria were applied for to the reference genes than to the target genes because inaccurate
276 reference gene measurements will affect every assay, whereas inaccurate target gene
277 measurements will only affect that single assay.

278 Standard curves, run in duplicate on both plates to give quadruplicates, were calculated from serial
279 dilutions (0.2x, 0.04x, 0.008x and 0.0016x) from a cDNA mixture of all samples that was made up

before the STA step (Bustin et al., 2009). The undiluted sample (1x) did not have a linear relationship with the other dilutions for any of the assays, and so was not used in the standard curve. When replicates had a range greater than 1 Ct, we excluded this dilution. If fewer than 3 dilutions remained, normally in association with high Ct values, we excluded the whole assay (*i.e.*, α_{1A} -adrenoceptor, β_2 -adrenoceptor, *HCN4b* and *NKA $\alpha 1b$*)

CALCULATING GENE EXPRESSION

$E^{-\Delta Ct}$ (Normalised Quantity)

$E^{-\Delta Ct}$, expression normalised to a housekeeping gene, was calculated for a semi-quantitative comparison of the expression of different target genes. This measure was obtained as in Scott et al. (2004) using the geometric mean of the reference genes as in Hellemans et al. (2007) (*Eqn. A1*).

$E^{-\Delta\Delta Ct}$ (Calibrated Normalised Quantity)

$E^{-\Delta\Delta Ct}$, expression of the target gene normalised to the expression of the reference genes then an inter-run calibrator sample (IRC), a 0.04x dilution of pooled sample (the highest standard curve measurement used), was calculated as in Pfaffl (2001) using the geometric means of the reference genes, as in (*Eqn A2*).

Fold Change in $E^{-\Delta\Delta Ct}$ with Warm Acclimation (Calibrated Normalised Relative Quantity)
Fold change in $E^{-\Delta\Delta Ct}$ of the target gene with warm acclimation was calculated for each pacemaker rate response group, by dividing $E^{-\Delta\Delta Ct 12^\circ C}$ (*Eqn A2*) by the $E^{-\Delta\Delta Ct average 4^\circ C}$ (*Eqn A3*), in a method that is mathematically identical to Hellemans et al. (2007).

STATISTICAL ANALYSIS OF mRNA EXPRESSION

PCA analysis was performed using the PRCOMP package in R Studio (Team, 2014) with 68% confidence ellipses used to depict one standard deviation. FDR adjustments by GraphPad Prism 7.00 (GraphPad Software; CA) and other statistical analysis was performed Sigmaplot 14.0 (Systat; CA).

Four primary questions were examined with our qRT-PCR study. First, how does mRNA expression differ among cardiac tissues? - by comparing the mRNA expression in the SAN, atrium and ventricle in fish acclimated to 4°C for at least 3 weeks (Experiment 1). Second, how does cardiac tissue mRNA expression change with thermal acclimation? - by comparing mRNA expression in fish acclimation to either 4°C or 12°C after at least 3 weeks (Experiment 1). Third, what changes in mRNA expression are specifically associated with pacemaker rate resetting? - by comparing fish in Experiment 1 and Experiment 2 that had been acclimated for more than 3 weeks to either 4°C or 12°C. To answer these question, genes were placed into one of three groups:

311 1) *mRNA changes associated with warm acclimation, but not pacemaker rate resetting*: For
312 these genes, the fold change in mRNA expression was significantly different between 4°C
313 and 12°C in Experiments 1 and 2, but not different with warm acclimation between the two
314 Experiments.

315 2) *mRNA changes only associated with pacemaker rate resetting*: For these genes, the fold
316 change in mRNA expression was significantly different between 4°C and 12°C only in
317 Experiment 1, which meant a significant difference with warm acclimation between the two
318 Experiments.

319 3) *mRNA changes associated with no pacemaker rate resetting*: between 4°C and 12°C, only
320 in Experiment 2, which meant a significant difference with warm acclimation between the
321 two Experiments.

322 Lastly, we examined if the mRNA expression changes associated with pacemaker rate resetting
323 during full thermal acclimation could also be driving the initial resetting of pacemaker rate by
324 comparing mRNA expression in control fish moved from 4°C to 4°C for 1 h, and experimental fish
325 moved from 4°C to 12°C for 1 h. The mRNA expression changes seen within this first hour were then
326 compared to those changes in mRNA only associated with pacemaker rate resetting.

327 COMPARISON OF mRNA EXPRESSION IN CARDIAC TISSUE 328 TYPES

329 Differences in mRNA expression patterns were compared between cardiac tissue types with principal
330 component analysis, using $E^{-\Delta\Delta Ct}$. A 68% confidence limit ellipse was plotted around each tissue type
331 to graphically illustrate one standard deviation for the distribution.

332 Significant differences in genes between cardiac tissues were identified by comparing $E^{-\Delta\Delta Ct}$ mRNA
333 expression using a one-way ANOVA with a Tukey's *post-hoc* test. If the test for normality (Shapiro-
334 Wilk) or equal variance (Levene's) failed, the non parametric Kruskal-Wallis ANOVA with a Dunn's
335 *post-hoc* test was run.

336 For mRNA expression of the membrane clock and calcium clock proteins, relative expression is as
337 important as overall expression. Therefore, pie charts were created to qualitatively compare the
338 relative expression for each tissue type from average $E^{-\Delta Ct}$ values for HCN expression (*Fig. S2*) and
339 calcium clock expression (*Fig. S3*).

340 A semi-quantitative measure of total mRNA expression for each functional gene group was
341 calculated from the total $E^{-\Delta Ct}$ values of all the genes in each group for each individual. These were

342 compared among cardiac tissues, using a one-way ANOVA with a Tukey's *post-hoc* test. If the test for
343 normality (Shapiro-Wilk) or equal variance (Levene's) failed, the non parametric Kruskal-Wallis
344 ANOVA with a Dunn's *post-hoc* test was run. FDR adjustment was performed within each thermal
345 acclimation group

346 COMPARISON OF mRNA EXPRESSION BETWEEN WARM- AND 347 COLD-ACCLIMATED FISH

348 Differences in mRNA expression patterns were compared between warm- and cold- acclimated fish
349 with principal component analysis, using $E^{-\Delta\Delta Ct}$. A 68% confidence limit ellipse was plotted around
350 each tissue type to graphically illustrate one standard deviation for the distribution.

351 Significant differences in genes between warm- and cold-acclimated fish were established by
352 comparing $E^{-\Delta\Delta Ct}$ mRNA expression, within tissue and treatment groups, with Student's t-test (Fenna,
353 2013; Hassinen et al., 2008; Korajoki and Vornanen, 2012). If the test for normality (Shapiro-Wilk) or
354 equal variance (Levene's) failed, the non-parametric Mann-Whitney test was run.

355 FDR adjustment was performed within each tissue for each temperature and thermal acclimation
356 group.

357 COMPARISON OF THE FOLD CHANGE IN mRNA EXPRESSION 358 WITH WARMING

359 Significant differences between the pacemaker rate resetting responses were established by
360 comparing the fold change in $E^{-\Delta\Delta Ct}$ with warm acclimation for mRNA expression between fish with
361 and without a pacemaker rate resetting response using Student's t-test. If the test for normality
362 (Shapiro-Wilk) or equal variance (Levene's) failed, the non-parametric Mann-Whitney test was run.

363 FDR adjustment was performed within each tissue for each thermal acclimation group.

364 CORRECTING FOR MULTIPLE COMPARISONS

365 Multiple comparisons increase the likelihood of false positive results. Therefore, false discovery rate
366 (FDR) adjustments were applied to all ANOVAs and Student's t-tests using a two-stage step-up
367 method (Benjamini et al., 2006). However, unadjusted *P*-values are also presented for comparison.

368 DATA PRESENTATION

369 Data presentation, including Venn diagrams for comparisons of cold- and warm-acclimation and
370 comparisons of the fold changes in mRNA expression with warm acclimation, was done using R

371 Studio (Team, 2014), GraphPad Prism 7.00 (GraphPad Software; CA) and Inkscape 0.92 (Inkscape
372 Team).

373 RESULTS

374 Pacemaker rate resetting

375 In Experiment 1, fish rapidly reset intrinsic heart rate during both warm acclimation and cold
376 acclimation (*Fig. 1*). When intrinsic heart rate was measured at 4°C, it was significantly higher after 4-
377 6 weeks in 4°C-acclimated fish (36.1 ± 0.9 bpm) compared with 12°C-acclimated fish (33.4 ± 0.7 bpm)
378 ($P=0.044$ Student's t-test) (*Table 1*). After transferring 4°C-acclimated fish to 12°C, resetting of the
379 pacemaker rate (from 56.8 ± 1.2 bpm to 50.8 ± 1.5 bpm) was first seen after 1 h, and the new rate
380 persisted through until the 24 h measurement (50.3 ± 1.5 bpm) (*Fig. 1*). Fish-handling control fish
381 transferred back to 4°C, however, maintained a heart rate of 57.9 ± 1.9 bpm after 1 h, confirming
382 that the pacemaker rate resetting was caused by the temperature change. Similarly, significant
383 pacemaker rate resetting (from 33.4 ± 0.7 bpm to 37.7 ± 1.2 bpm) was seen when 12°C-acclimated
384 fish were moved to 4°C, but this was first detected after 8 h, rather than 1 h, and again persisted
385 unchanged for the 24 h measurement (37.7 ± 1.2 bpm) (*Fig. 1*).

386 In Experiment 2, fish did not reset pacemaker rate with either temperature acclimation. When 4°C-
387 acclimated fish were moved to 12°C, intrinsic heart rate was unchanged after both 24 h (52.7 bpm \pm
388 2.2 bpm vs 57.5 ± 2.7 bpm) and 10-12 weeks (53.3 ± 2.8 bpm vs 52.2 ± 2.3 bpm) (*Table 1*). Similarly,
389 when 12°C-acclimated fish were moved to 4°C, intrinsic heart rate was unchanged after both 24 h
390 (30.6 ± 1.7 bpm vs 29.2 ± 0.6 bpm) and 10-12 weeks (29.9 ± 1.1 bpm vs 29.4 ± 1.1 bpm) (*Table 1*).

391 mRNA expression

392 mRNA EXPRESSION DIFFERENCE BETWEEN TISSUES

393 ACCLIMATED TO 4°C FOR MORE THAN 3 WEEKS

394 Principal component analysis revealed mRNA expression in the ventricle was distinct from both SAN
395 and atrium. SAN and atrium had a minor overlap of 68% confidence intervals for PC1, and PC1 and
396 PC2 explained 35.9% and 19.4% of variance in gene expression, respectively (*Fig. 2-A*), perhaps in
397 part due to the minor atrial content of the SAN sample. Nevertheless, mRNA expression in the SAN
398 and atrium did differ specifically for the membrane clock component *HCN1*, the calcium clock
399 component *Ca_v1.3* and *collagen $\alpha 1a$* (*Fig. 3*). Furthermore, the mRNA expression of membrane clock
400 proteins was highest for SAN (*Fig. S2*), unlike the calcium handling protein mRNA expression (*Fig. S3*)
401 which were not significantly different among the three cardiac tissues.

402 mRNA EXPRESSION CHANGES AS A RESULT OF WARM 403 ACCLIMATION

404 Principal component analysis also revealed differences in mRNA expression between warm
405 acclimation and cold acclimation; PC1 and PC2 explained 24.0% and 22.1% of variance in gene
406 expression, respectively (Fig. 2-B). Warm acclimation and pacemaker rate resetting in Group 1 was
407 associated with differential expression of 20 genes: 6 in SAN, 5 in atrium and 9 in ventricle (Fig. 4).
408 Specifically, a membrane clock component (*HCN2a2*) was significantly downregulated with warm
409 acclimation in the SAN, but not in the atrium (Fig. 4). Notably, *HCN4a1/2* was the dominant HCN
410 isoform and was upregulated relative to total HCN expression in all three tissue types (Fig. S2). Warm
411 acclimation also downregulated mRNA expression of calcium clock components *calsequestrin* and
412 *SERCA2* in both SAN and atrium, while mRNA expression of *S100* was downregulated only in SAN and
413 mRNA expression of *RYR3* was downregulated only in the atrium (Fig. 4). mRNA expression of *NKA*
414 $\alpha 1a$ and *NKA* $\alpha 1c$ were also downregulated in both SAN and atrium. However, mRNA expression of
415 *K_{ir}2.1* and *K_{ir}2.2*, *ERG*, *Cx43* and *collagen 1 α 1* did not change significantly in either atrium or ventricle,
416 and neither *SERCA2* or *calsequestrin* were downregulated in the ventricle for fish that reset
417 pacemaker rate (Fig. 4).

418 mRNA EXPRESSION CHANGES ASSOCIATED WITH 419 PACEMAKER RATE RESETTING

420 Of the 20 gene expression changes associated with warm acclimation, 9 fitted the criteria for 'mRNA
421 changes only associated with pacemaker rate resetting' (*i.e.*, they were observed only in Experiment
422 1) (Fig. 4, Fig. 5, Fig. 6 and Fig. S4). Specifically, downregulation of calcium clock components *S100*
423 and *SERCA2* mRNA expression occurred only in SAN, downregulation of *calsequestrin* mRNA
424 expression occurred only in atrium, and upregulation of *HCN1*, *HCN4a1/2* and *NKA* $\alpha 3$ mRNA
425 expression occurred only in ventricle. Downregulation of *NKA* $\alpha 1c$ expression with warm acclimation
426 was the only change common to SAN, atrial and ventricular tissue that was unique to the fish in
427 Experiment 1 (Fig. 4, Fig. 5 and Fig. S4). However, after FDR adjustment, only the upregulation of
428 *HCN1* in ventricle and the downregulation of *NKA* $\alpha 1c$ in atrium remained significant (Fig. 4, Fig. 5,
429 Fig. 6 and Fig. S4).

430 Of the 14 gene expression changes observed with warm acclimation in Experiment 2 (5 in the SAN, 6
431 in the atrium and 3 in the ventricle), 6 fitted the criteria for 'mRNA changes associated with no
432 pacemaker rate resetting' (*i.e.*, they were observed only in Experiment 2) (Fig. 6). Specifically,
433 upregulation of membrane clock component *HCN3* and *TGF- β 1* occurred in both SAN and atrium,
434 downregulation of *HCN2a1* and *HCN2a2* and an upregulation of *collagen 1 α 1* occurred only in
435 atrium, and a downregulation of *calsequestrin* occurred only in ventricle (Fig. 4, Fig. 5 and Fig. S4).

436 However, after FDR adjustment only the upregulation of membrane clock component *HCN3* in SAN
437 and atrium remained significant (Fig. 4, Fig. 5, Fig. 6 and Fig. S4).

438 Gene expression changes that fitted the criteria for 'mRNA changes associated with warm
439 acclimation, but not pacemaker rate resetting' (Fig. 6) included downregulation of the membrane
440 clock component *HCN2a2* and the calcium clock component *calsequestrin* mRNA expression in SAN,
441 downregulation of *RYR3* mRNA expression in atrium, and an upregulation of *HCN3* mRNA expression
442 in ventricle (Fig. 4, Fig. 5 and Fig. S4). However, after FDR adjustment none of these expression
443 changes remained significant (Fig. 4, Fig. 5, Fig. 6 and Fig S4).

444 mRNA expression changes observed 1 h after warm acclimation in fish with a pacemaker rate
445 resetting response (Fig. 7) included upregulation the membrane clock component *HCN2b1/2* and
446 calcium clock component *NCX* in SAN, downregulation of *TGF- β 1* in SAN and atrial tissues,
447 upregulation of *calsequestrin* in atrium, upregulation of *Na_v1.5* in ventricle and downregulation of
448 *TRPC1* in ventricle. However, none of these changes were significant after FDR adjustment. None of
449 these genes showed expression patterns that fitted the criteria for 'mRNA changes only associated
450 with pacemaker rate resetting' in the fish acclimated for > 3 weeks (Fig. 6, Fig. 7).

451 DISCUSSION

452 Before our study was undertaken, it was assumed that thermal acclimation was a typical
453 homogenous process within species, and differences observed in pacemaker resetting response to
454 thermal acclimation were species-specific (Matikainen and Vornanen, 1992). However, unexpected
455 results from our study demonstrated that pacemaker resetting does not always occur in rainbow
456 trout. While Experiment 1 showed intrinsic heart rate resetting in response both warm and cold
457 acclimation, Experiment 2 did not show intrinsic heart rate resetting, even after 10 weeks
458 acclimation to either 4°C or 12°C. This means that the lack of pacemaker resetting responses in fishes
459 cannot be attributed solely to species differences, as suggested previously by Matikainen and
460 Vornanen, 1992. What might turn off pacemaker resetting awaits further study, but the present
461 results suggest seasonal acclimatisation or previous life experiences could be a factor. Indeed
462 differences in both intrinsic heart rate, and in the electrophysiology of cardiac myocytes have been
463 previously observed in a number of fish species, although not in salmonids (Filatova et al., 2019;
464 Harri and Talo, 1975; Matikainen and Vornanen, 1992). A severe hypoxic stress event is one
465 experience that could affect intrinsic heart rate resetting in rainbow trout (Sutcliffe, 2018). Even so,
466 our two experimental groups were from different brood years with different life experiences and
467 sampled at different times of year, all of which could contribute to the variation in the pacemaker
468 resetting response to thermal acclimation seen here.

469 The second novel discovery from our study was the speed at which intrinsic heart rate resetting can
470 occur, which we quantified by reciprocally transferring rainbow trout to either 4°C or 12°C. In the
471 fish with a pacemaker resetting response (Experiment 1), intrinsic heart rate was significantly
472 different after just 1 h of warm acclimation to 12°C, and after just 8 h of cold acclimation to 4°C. The
473 changes in heart rate were in the direction expected, adding confidence to our novel finding rapid
474 intrinsic heart rate resetting. Furthermore, the change in intrinsic heart rate was faster with warm
475 acclimation, as expected, and most likely as a result of faster reaction rates at higher temperatures.
476 Similarly, changes in CT_{max} and CT_{min} in sheepshead minnow were more rapid for warm vs cold
477 acclimation (Fangue et al., 2014).

478 Few studies have explicitly examined the timescale to reset intrinsic heart rate with temperature
479 acclimation; they typically examine heart rate after many days or weeks. For example, heart rate
480 resetting was observed after 4-10 days in American bullfrogs and wood frogs (Lotshaw, 1977), after 2
481 weeks in the common frog (Harri and Talo, 1975), after 3 weeks in perch (Talo and Tirri, 1991), after
482 4 weeks in rainbow trout (Aho and Vornanen, 2001) and after 2 months in goldfish (Hiroko et al.,
483 1985; Tsukuda, 1990). Only one study (Ekstrom et al., 2016) examined the time course of pacemaker
484 resetting between 1 day and 6 weeks after transfer from 9°C to 16°C and in rainbow trout. However,
485 they observed of no intrinsic heart rate resetting. While they concluded that pacemaker rate
486 resetting takes longer than 6 weeks, our study offers two alternative explanations that likely depend
487 the specific population or group of rainbow trout: a) intrinsic heart rate may not reset, like our
488 Experiment 2; or b) the resetting had been completed by their first measurement at 24 h, like our
489 Experiment 1. Whatever the explanation, our study demonstrates that pacemaker resetting can be
490 significantly faster rate than previously assumed, within hours rather than weeks, and even with cold
491 acclimation.

492 Pacemaker resetting is not the only cardiac response to thermal acclimation. While not the focus of
493 our study, cardiac thermal acclimation responses identified in numerous other studies include
494 decreases in the stiffness of the ventricle tissues, in the weight of the ventricle relative to body size,
495 in cardiac force, in the density of cardiac β -adrenergic receptors and in the cardiac sensitivity to β -
496 adrenergic stimulation (Aho and Vornanen, 1999; Aho and Vornanen, 2001; Altimiras and Axelsson,
497 2004; Anttila et al., 2013; Brett, 1971; Casselman et al., 2012; Chen et al., 2013; Chen et al., 2015;
498 Costa et al., 2002; Drost et al., 2014; Eliason et al., 2011; Eliason et al., 2013; Farrell, 1991; Fry, 1947;
499 Gilbert et al., 2019; Graham and Farrell, 1989; Keen et al., 1993; Keen et al., 2016; Keen et al., 2017;
500 Klaiman et al., 2011; Klaiman et al., 2014; Shiels and Farrell, 1997; Steinhausen et al., 2008; Verhille
501 et al., 2013; Wood et al., 1979). Whether these responses are homogenous with thermal acclimation
502 or even whether they occurred in our study are unknown. However, we did observe some mRNA
503 expression changes in atrial and ventricle tissue that might be related to the previous studies that

504 have examined mRNA expression in atrial and in ventricle tissue during warm acclimation to identify
505 what mRNA expression changes might be causing these responses. For example, similar to Hassinen
506 et al. (2007) and by Korajoki and Vornanen (2012 and 2009), fish with an intrinsic heart rate resetting
507 response had no change in *K_{ir}2.1* and *K_{ir}2.2* expression, a downregulation in *SERCA2* in atrial and
508 ventricle tissue, and a downregulation of *calsequestrin* in only atrial tissue during warm acclimation.
509 However, other previously observed differences in mRNA expression with warm acclimation that we
510 did not observe, *i.e.* *ERG*, *Cx43* and *collagen 1 α 1* (Fenna, 2013; Hassinen et al., 2008). Whether
511 this difference reflects a different experimental temperatures among studies (4°C and 12°C in our
512 study vs 4°C and 18-19°C previously), or some other variable is unclear. For fish that did not reset
513 intrinsic heart rate, downregulation in *SERCA2* was not observed, downregulation of *calsequestrin*
514 was observed only in the ventricle and an upregulation of collagen α 1a was observed in the atrium.
515 While previous studies report other cardiac changes during thermal acclimation, heart rate was not
516 measured in parallel with mRNA expression, something that we feel should be done to confirm
517 resetting.

518 We identifies a number of novel mRNA expression changes in the atrium and ventricles of the fish
519 that reset intrinsic heart rate, as well as in those that did not. Resetting of intrinsic heart rate with
520 acclimation was associated with downregulation of *NKA α 1c* and *RYR3* was observed in atrium and
521 ventricle, a downregulation of *NKA α 1a* in the atrium, a downregulation of *NCX* in the ventricle, and
522 an upregulation in *NKA α 1a*, *Nav1.6*, *HCN1*, *HCN3* and *HCN4a1/2* in the ventricle of the. Of these
523 changes only the downregulation in the ventricle of *RYR3* and upregulation in the ventricle of *HCN3*
524 was observed also in the fish that did not reset intrinsic heart rate. Other changes observed in the
525 atrium of fish that didn't reset intrinsic heart rate were downregulation of *HCN2a1* and *HCN2a2*, and
526 upregulation of *HCN3*, and *TGF- β 1*, as well as an upregulation of *HCN 2b1* in the ventricle. What role
527 these novel changes play in thermal acclimation is unclear as in this study we did not confirm any
528 differences in cardiac thermal acclimation responses between the two groups of fish other than in
529 intrinsic heart rate resetting.

530 SAN, not ventricle or atrial, tissues is responsible for resetting pacemaker rate. Therefore, mRNA
531 expression differences between SAN tissue and other cardiac tissues such as the atrium likely reflect
532 a role in pacemaking. However, since the SAN occupies a small region of the heart and cannot be
533 easily defined by eye, we conservatively dissected the SAN tissue to reduce the risk of accidentally
534 missing SAN tissue, but this meant that a minimal portion of atrial tissue was included. Despite this
535 potential contamination, mRNA expression patterns seen in the SAN were not necessarily seen in
536 atrium, and vice versa. For example, *HCN1*, *collagen 1 α 1*, and *Ca_v1.3* were differentially expressed
537 between the atrial and SAN tissues. Therefore, the mRNA expression ascribed solely the SAN tissue is

538 done with a good measure of confidence and, if anything, any expression differences identified
539 would potentially be underestimated.

540 Our is the first in fishes to examine the mRNA expression of components of the calcium and the
541 molecular clock in the SAN. Both clocks are hypothesised to drive spontaneous depolarisation of
542 pacemaker cells in the SAN in mammals, and evidence exists that may (Hassinen et al., 2017;
543 Marchant and Farrell, 2019) or may not be the case in fishes (Wilson and Farrell, 2013). Therefore,
544 the differentially higher expression of *HCN1*, a molecular clock component, observed in SAN
545 compared with atrial tissues is of significant interest. This is similar to observations in mammals
546 (Marionneau et al., 2005) where *HCN1* is the fastest activating HCN isoform and is activated at the
547 highest voltage (Wahl-Schott and Biel, 2009). If rainbow trout *HCN1* has similar properties, then its
548 significantly increased expression in the SAN could mean that the molecular clock is responsible for
549 the SAN's rapid rate of depolarisation. This, along with physiological experiments demonstrating that
550 HCN antagonists do slow down heart rate *in vivo* in rainbow trout (Altimiras and Axelsson, 2004;
551 Keen and Gamperl, 2012), and calcium antagonists have only small effects on heart rate (Haverinen
552 and Vornanen, 2007), lends support to a membrane clock theory of spontaneous depolarisation in
553 rainbow trout. However, a high *HCN1* mRNA expression does not necessarily correlate with a
554 functional role in the spontaneous depolarisation of pacemaker cells. Also, it was the *HCN4* isoform
555 that dominated in all cardiac tissues from rainbow trout. Furthermore, in brown trout, cardiac
556 pacemaker cells have a high HCN expression, but no I_f current, suggesting a membrane clock may not
557 function in the spontaneous depolarisation of pacemaker cells in this species (Hassinen et al., 2017).
558 Thus, further work is needed to resolve the mechanism responsible for spontaneous depolarisation
559 in salmonids, and further studies of mRNA expression may not be the best way forward.

560 Key to understanding what drives pacemaker rate, is understanding what causes it to reset. Despite
561 this, changes in mRNA expression with temperature acclimation have not been previously examined
562 in SAN of any fish. If *HCN1*, and other molecular clock components set the pacemaker rate, we would
563 expect changes in expression of these isoforms in SAN after warm acclimation in fish that reset
564 intrinsic heart rate. However, we observed very few mRNA expression changes that were unique to
565 the SAN. These were restricted to downregulation of *HCN2a2* and *s100*, components of the
566 membrane clock and the calcium clock, respectively. Conversely, mRNA expression in a different
567 calcium clock component, *calsequestrin*, was observed unique to the SAN of fish that did not reset
568 intrinsic heart rate. However, if a mRNA expression change is associated with heart resetting, it will
569 probably would not only be observed in fish with a pacemaker rate resetting response, it would also
570 be unique to those fish. Identifying these changes was greatly aided by comparisons between fish in
571 Experiment 1 and fish in Experiment 2. These would be genes with significantly different mRNA
572 expression between warm- and cold-acclimated fish, only in fish with a pacemaker rate resetting

573 response, along with a greater fold change in mRNA expression changes with warm acclimation. Of
574 these changes the genes most likely to be responsible are those which occurred in the SAN such as
575 *NKA $\alpha 1c$* , *S100* and *SERCA2*. However, no mRNA expression changes unique to the fish that reset
576 intrinsic heart rate were significant after FDR adjustment. Therefore, we did not identify any strong
577 links between mRNA expression and intrinsic heart rate resetting.

578 This leads back to the rapid pacemaker resetting response observed *in vitro*. None of the mRNA
579 expression that were unique to the fish that reset intrinsic heart rate with >3 weeks acclimation were
580 present in the fish after just 1 h of warm acclimation. While changes in mRNA expression might not
581 be expected to occur so rapidly, this lack of association does suggest that the initial and rapid
582 resetting of pacemaker rate, and perhaps pacemaker resetting in general, is not driven by changes in
583 mRNA expression. Therefore, future studies of pacemaker setting in fishes should consider
584 alternative mechanisms, such as post-transcriptional protein modifications.

585 In conclusion, pacemaker rate resetting with thermal acclimation occurred much more rapidly than
586 previously shown in any ectotherm, even though we also showed thermal acclimation can occur in
587 rainbow trout without pacemaker rate resetting. The rapid pacemaker rate resetting response,
588 however, was not associated with any significant changes in mRNA expression for key proteins in the
589 SAN, and we did not identify any mRNA expression changes that that would be a strong candidate to
590 be driving the pacemaker resetting response. Nevertheless, differential changes in mRNA expression
591 during thermal acclimation were discovered among SAN, atrial and ventricular, including novel
592 candidate genes in SAN tissue that are associated with warm acclimation but independent of
593 resetting pacemaker rate.

594 LIST OF SYMBOLS AND ABBREVIATIONS

°C	Degrees Celsius
µg	Microgram
µL	Microlitre
µM	Micromolar
18s	18s ribosomal RNA
ANOVA	Analysis of variance
ANP	Atrial natriuretic peptide
bpm	Beats per minute
cAMP	Cyclic adenosine monophosphate
Ca _v 1.3	L-type calcium voltage-gated channels subunit $\alpha 1D$

Ca _v β2	L-type calcium voltage-gated channels subunit β2
CCDC84	Coiled-coil domain containing 84
cDNA	Complementary deoxyribonucleic acid
Col1α1	Collagen type 1a1
Ct	Cycle threshold
CT _{max}	Critical thermal maximum
CT _{min}	Critical thermal minimum
Cx43	Connexin 43
DnaJA2	DnaJ heat shock protein family (Hsp40 member A2)
EF1-α	Elongation factor 1-α
ERG	Ether-à-go-go related gene
E ^{-ΔCt}	Expression normalised to reference genes
E ^{-ΔΔCt}	Expression normalised to reference genes and inter-run calibrators
FDR	False discovery rate
g	Gram
h	Hour
HCN1	Hyperpolarisation-activated cyclic nucleotide-gated channel 1
HCN2a1	Hyperpolarisation-activated cyclic nucleotide-gated channel 2a1
HCN2a2	Hyperpolarisation-activated cyclic nucleotide-gated channel 2a2
HCN2b1	Hyperpolarisation-activated cyclic nucleotide-gated channel 2b1
HCN2b1/2	Hyperpolarisation-activated cyclic nucleotide-gated channel 2b1/2
HCN3	Hyperpolarisation-activated cyclic nucleotide-gated channel 3
HCN4a1/2	Hyperpolarisation-activated cyclic nucleotide-gated channel 4a1/2
HCN4b	Hyperpolarisation-activated cyclic nucleotide-gated channel 4b
IRC	Inter-run calibrator
K _{ir} 2.1	Potassium voltage-gated channel subfamily J member 2
K _{ir} 2.2	Potassium voltage-gated channel subfamily J member 12
L	Litre
M	Molar
mg	Milligram
min	Minute
mL	Millilitre
mm	Millimetre
mM	Millimolar

mRNA	Messenger ribonucleic acid
Mrpl40	39s ribosomal protein L40
n	Sample size (number of animals or tissue samples in each group)
Na _v 1.4	Sodium voltage-gated channel α subunit 4
Na _v 1.5	Sodium voltage-gated channel α subunit 5
Na _v 1.6	Sodium voltage-gated channel α subunit 8
NCX	Sodium-calcium exchanger
NKA α1a	Sodium/potassium ATPase subunit α1a
NKA α1b	Sodium/potassium ATPase subunit α1b
NKA α1c	Sodium/potassium ATPase subunit α1c
NKA α3	Sodium/potassium ATPase subunit α3
P-value	Probability value
pg	Picogram
Q ₁₀	Temperature coefficients
qRT-PCR	Quantitative real-time polymerase chain reaction
RYR3	Ryanodine receptor 3
s	Second
S100	S100 calcium binding protein
SAN	Sinoatrial node
sem	Standard error of the mean
SEP15	15 kDa selenoprotein
SERCA2	Sarcoplasmic/ endoplasmic reticulum calcium ATPase 2
TGF-β1	Transforming growth factor β1
TRPC1	Transient receptor potential cation channel subfamily C member 1
UBC	University of British Columbia

595

596 APPENDIX

597

598 *Equation A1:*
$$\frac{E(target)^{-Ct(target)}}{(E(CCD C84)^{-Ct(CCD C84)} \times E(SEP15)^{-Ct(SEP15)})^{\frac{1}{2}}}$$

599 *Equation A2:*
$$\frac{E(target)^{\Delta Ct(sample - IRC(target))}}{(E(CCD C84)^{-\Delta Ct(sample - IRC(CCD C84))} \times E(SEP15)^{-\Delta Ct(sample - IRC(SEP15))})^{\frac{1}{2}}}$$

600

Equation A3:

$$\frac{E(target)^{-\Delta Ct_2(arithmetic\ mean\ 4^{\circ}C - IRC(target))}}{(E(CDC84)^{-\Delta Ct_2(arithmetic\ mean\ 4^{\circ}C - IRC(CDC84))} \times E(SEP15)^{-\Delta Ct_2(arithmetic\ mean\ 4^{\circ}C - IRC(SEP15))})^{\frac{1}{2}}}$$

601 **Table A1 - All primer pairs used for Fluidigm qRT-PCR analysis.**

602 All primers were designed specifically for this study from the sequence given by the accession number or copied directly from the study, unless indicated
603 otherwise. Primers are displayed in the 5' to 3' direction.

Target	Forward Primer	Reverse Primer	Accession Number/ Source Study
<i>18s</i> (Reference Gene)	CGGTCGGCGTCCAACTT	CAATCTCGCGTGGCTGAA	AF243428
<i>β-actin</i> (Reference Gene)	TGGGGCAGTATGGCTTGATG	CTGGCACCTAATCACTCT	Ojima (2007)
<i>CCDC84</i> (Reference Gene)	GCTCATTTGAGGAGAAGGAGGATG	CTGGCGATGCTG TTCCTGAG	Jeffries et al. (2014)
<i>DnaJA2</i> (Reference Gene)	TTGTAATGGAGAAGGTGAGG	TGGGCCGCTCTCTTGATGT	Hassinen et al. (2007)
<i>EF1-α</i> (Reference Gene)	ACCCTCCTCTTGGTCGTTT	TGATGACACCAACAGCAACA	Raida and Buchmann (2007)
External standard (Reference Gene)	GTGCTGACCATCCGAG	GCTTGTCGGTATAACT	Ellefsen et al. (2008)
<i>Mrpl40</i> (Reference Gene)	CCCAGTATGAGGCACCTGAAGG	GTAAATGCTGCCACCCTCTCAC	Jeffries et al. (2014)
<i>SEP15</i> (Reference Gene)	TCACAGCAAACCACATTTTGG	AAGATGCCCAGAGTGACACACA	AY255833
<i>Hyperpolarisation-activated cyclic nucleotide-gated channel 1 (HCN1)</i>	CGCTGAGGATCGTGAGGTTT	TGAGCCGGGAAAGTCTCAGT	AF421883
<i>Hyperpolarisation-activated cyclic nucleotide-gated channel 2a1 (HCN2a1)</i>	ATCGTGGACTTTGTCTCCTCCAT	GATCCCCTTCTCCACGATCA	XM_014141001
<i>Hyperpolarisation-activated cyclic nucleotide-gated channel 2a2 (HCN2a2)</i>	CTGCAGGACTTCCCCTCAGA	CCAGGTGTCATTACCATCTTG	XM_014191309
<i>Hyperpolarisation-activated cyclic nucleotide-gated channel 2b1 (HCN2b1)</i>	CGCCAGTACCAGGAGAAGTACA	AGCTGGCAGTTTGTGGAAAGA	XM_014148806

<i>Hyperpolarisation-activated cyclic nucleotide-gated channel 2b1/2 (HCN2b1/2)</i>	CCAGTGCAGTGATGCGTATCTT	ACAGCCATCCCAGTGACACA	AY148882
<i>Hyperpolarisation-activated cyclic nucleotide-gated channel 3 (HCN3)</i>	ACGGACGTATGGCTGACTATCA	CCGGAAACATGGCATAGCA	XM_014176849
<i>Hyperpolarisation-activated cyclic nucleotide-gated channel 4a1/2 (HCN4a1/2)</i>	TGGGAGGAGATCTTCCATATGAC	CAGGTTGACGATACGCACCAT	XM_014175391 and XM_014126754.1
<i>Hyperpolarisation-activated cyclic nucleotide-gated channel 4b (HCN4b)</i>	CGGGCGCTGAGAATCGT	CGGAGCAACCTCAACAAACTC	XM_014170228
<i>L-type calcium voltage-gated channels subunit $\alpha 1D$ ($Ca_v1.3$)</i>	CGGCAAGTCGCCAAGT	GCGGAGCGTGCTCGTAGTAG	NM_001124328
<i>L-type calcium voltage-gated channels subunit $\beta 2$ ($Ca_v\beta 2$)</i>	TGACATAGATGCCACAGGCTTAGA	GAGCGGAGGTGGACTGGAA	DQ198264
<i>Calsequestrin</i>	CCAACCCTACATCAAATCTTTGC	TCATTTTCAGGGTCAGCTCCTT	NM_001160499
<i>Sodium-calcium exchanger (NCX)</i>	GCAATGCCGTCAACGTCTT	GGTAGATGGCAGCGATGGA	NM_001124598
<i>Ryanodine receptor 3 (RyR3)</i>	AGGCTTCCTCGGCTTTCAC	TGTCGGAAGTTGGAGATCTTCTT	EF032937
<i>Sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (SERCA2)</i>	GTGCTCGTCACGATAGAGATGTG	CAGCAGGGACTGGTTCTCTGA	Primers designed off sequences from Korajoki and Vornanen (2012)
<i>S100 calcium binding protein (S100)</i>	GTCAAGACTGGAGGCTCAGAG	GATCAAGCCCCAGAAGTGTTTG	Jeffries et al., 2014
<i>Transient receptor potential cation channel subfamily C member 1 (TRPC1)</i>	GAAGCGGAAGCGTGATGAG	GGTAGCGGTGGACAAGACAAC	NM_001185053
<i>Ether-à-go-go-related gene (ERG)</i>	TCCACGACGCACGAGAAAC	ACACGACTGGTCTGGAAATGAGT	NM_001124676
<i>Potassium voltage-gated channel subfamily J</i>	GCCCCAGAGCCGCTTT	AGAGACATTGATGAACTGCACGTT	DQ435674

<i>member 2 (K_{ir}2.1)</i>			
<i>Potassium voltage-gated channel subfamily J member 12 (K_{ir}2.2)</i>	CTACGGCTACCGCTGTGTGA	GGACTGGAAGACCACCATGAA	DQ435676
<i>Sodium voltage-gated channel α subunit 4 (Na_v1.4)</i>	TGTGCTCCGAGCCCTTAAA	CGCCCAATGGTTTTTCAG	EF203231
<i>Sodium voltage-gated channel α subunit 5 (Na_v1.5)</i>	TGCCACCCCTGCTGGTA	CGTGGACTGCTTTCCAGAT	EF203232
<i>Sodium voltage-gated channel α subunit 8 (Na_v1.6)</i>	CGACACCTTCACCTGCAACA	CATTGGTGACGAAGTCGTTCA	EF203233
<i>Sodium/potassium ATPase subunit α1a (NKA α1a)</i>	CCTTGGATGAGCTTAACAGGAAA	GCCCGAACCGAGGATAGAC	AY319391
<i>Sodium/potassium ATPase subunit α1b (NKA α1b)</i>	CCCATGGATTGCTGGGTAT	CCTCCATGTCGTTTCATTATCTTGT	AY319390
<i>Sodium/potassium ATPase subunit α1c (NKA α1c)</i>	TGCCAGAGGTATTGTCATCAACAC	GAGGCGAGGGTAGCAATACG	AY319389
<i>Sodium/potassium ATPase subunit α3 (NKA α3)</i>	GCCAGCAATGGACATATGAACA	ACTGACGAAGAAGGCTGTGTGA	AY319388
<i>α_{1A} adrenoceptor</i>	TGGGCTCTGTCTGTCACAATCT	AGGCGCCGGCTCCTT	NM_001124653
<i>α_{1B} adrenoceptor</i>	ACCGAAGAACCGGGCTATG	CATAAGCGGCAGGTAGAAGGA	NM_001124650
<i>β₂ adrenoceptor</i>	GCCTAAGCCCCAAGGACAAG	AGCTCCACGGTCCCAACAT	NM_001124440
<i>ANP precursor</i>	TCTGCTCCTGCTTGTCAACA	AGGGTATGGTCTGCCAACA	NM_001124211
<i>Collagen type 1a (Col1α1)</i>	CCCAGCCATGCCAGAT	CAGATAACTTCGTCGCACATGAC	NM_001124177
<i>Connexin 43 (Cx43)</i>	TGGCAGCACCATCTCCAA	GGGTGTCGTGAGGGAAGTCA	NM_001124563
<i>Transforming growth factor β1 (TGF-β1)</i>	GGGCTGGAAGTGGATCCAT	GGGCCGATGCAGTAGTTAGC	KF870471

604

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608 COMPETING INTERESTS

609 No competing interests compared

610 AUTHOR CONTRIBUTIONS

611 R.L.S collected and analysed the heart rate data and designed and tested the qRT-PCR assays at UBC.
612 The Fluidigm qRT-PCR was performed at the Pacific Biological Station, Fisheries and Oceans by S.L,
613 with the assistance of R.L.S, under the supervision of K.M.M. Editorial and technical feedback was
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809

810 **FIGURE LEGENDS**

811 **Figure 1 – Changes in intrinsic heart rate over time in response to an acute temperature change,**
812 **for fish from Experiment 1 (performed in the winter)**

813 Red symbols are for fish acutely transferred from 4°C- to 12°C with intrinsic heart rate
814 measurements being made at 12°C (n=6-9) while blue symbols are for fish acutely transferred from
815 12°C to 4°C with intrinsic heart rate measurements being made at 4°C (n=4-6).

816 The data are presented as mean ± SEM. Dissimilar letters are used to indicate statistically significant
817 differences between time points within an experimental group (one-way ANOVA).

818 **Figure 2 – Principal component analysis of mRNA expression of cardiac function genes.**

819 (A) Principal component analysis plot of sinoatrial node, atrial and ventricle mRNA expression of
820 cardiac function genes, in fish acclimated to 4°C for more than 3 weeks from Experiment 1 (n=7). All
821 E-ΔΔCt shown in Figure 3 were used in the PCA analysis, and plots were created from PC1 and PC2
822 which explained 35.9% and 19.4% of variance respectively. 68% confidence limit ellipses were
823 plotted around each tissue type. (B) Principal component analysis plot of mRNA expression of cardiac
824 function genes, in the SAN of fish acclimated to 4°C and 12°C from Experiment 1 (n=7). All E-ΔΔCt
825 shown in Figure 6A were used in the PCA analysis, and plots were created from PC1 and PC2 which

826 explained which explained 24.0% and 22.1% of variance respectively. 68% confidence limit ellipses
827 were plotted around each tissue type. Component Loadings are given in Table S2.

828 **Figure 3 – Sinoatrial node, atrial and ventricle mRNA expression of cardiac function genes in fish**
829 **acclimated to 4°C for more than 3 weeks from Experiment 1.**

830 Expression ($E^{-\Delta\Delta Ct}$) is normalised first to the geometric mean of the reference genes, *CCDC84* and
831 *SEP15*, and then an inter-run calibrator, and presented as mean \pm SEM (n=7). Table shows the
832 statistical comparisons between cardiac tissues for the data displayed in the graph on the right. * is
833 used to indicate statistically significant differences with FDR adjustment (<0.05)

834 **Figure 4 – mRNA expression of cardiac function genes, in the SAN (A), atrium (B), and ventricle (C)**
835 **of fish acclimated to 4°C and 12°C for more than 3 weeks for fish from Experiment 1.**

836 Expression ($E^{-\Delta\Delta Ct}$) is normalised to the geometric mean of the reference genes, *CCDC84* and *SEP15*,
837 then an inter-run calibrator and presented as mean \pm SEM (n=7 except in those examples specified in
838 the methods) * is used to indicate statistically significant differences with FDR adjustment (<0.05),
839 and (*) is used to indicate statistically significant differences without FDR adjustment (<0.05)
840 between 4°C and 12°C acclimations (Student's t test or Mann-Whitney test)

841 **Figure 5 – mRNA expression of cardiac function genes, in the SAN (A), atrium (B) and ventricle (C)**
842 **of fish acclimated to 4°C and 12°C for more than 3 weeks from Experiment 2.**

843 Expression ($E^{-\Delta\Delta Ct}$) is normalised to the geometric mean of the reference genes, *CCDC84* and *SEP15*,
844 then an inter-run calibrator and presented as mean \pm SEM (n=7) * is used to indicate statistically
845 significant differences with FDR adjustment (<0.05), and (*) is used to indicate statistically significant
846 differences without FDR adjustment (<0.05) between 4°C and 12°C acclimations (Student's t test or
847 Mann-Whitney test).

848 **Figure 6 - Venn Diagrams showing mRNA expression changes after more than 3 weeks of warm**
849 **acclimation that occur only in fish that reset intrinsic heart rate (Experiment 1), that only occur in**
850 **fish that don't reset intrinsic heart rate (Experiment 2) and in both groups of fish.**

851 This figure combines data from Fig. 4, Fig. 5 and Fig. S4.

852 **Figure 7 – mRNA expression of cardiac function genes, in the SAN (A), atrium (B) and ventricle (C)**
853 **of fish acclimated to 4°C and 12°C for 1 hour with an intrinsic heart rate resetting response.**

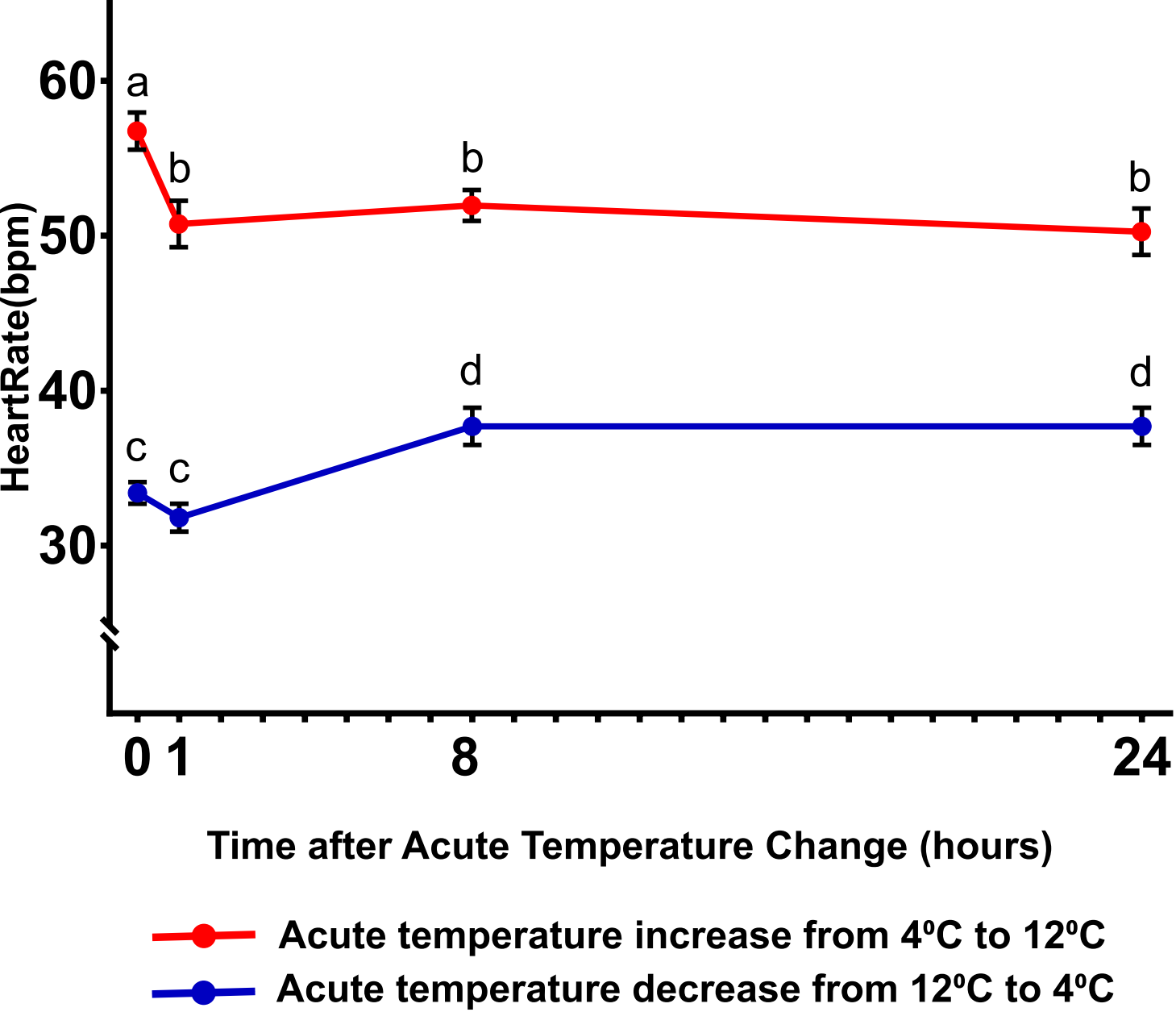
854 Expression ($E^{-\Delta\Delta Ct}$) is normalised to the geometric mean of the reference genes, *CCDC84* and *SEP15*,
855 then an inter-run calibrator and presented as mean \pm SEM (n=7) * is used to indicate statistically
856 significant differences with FDR adjustment (<0.05), and (*) is used to indicate statistically significant
857 differences without FDR adjustment (<0.05) between 4°C and 12°C acclimations (Student's t test or
858 Mann-Whitney test).

859 TABLES

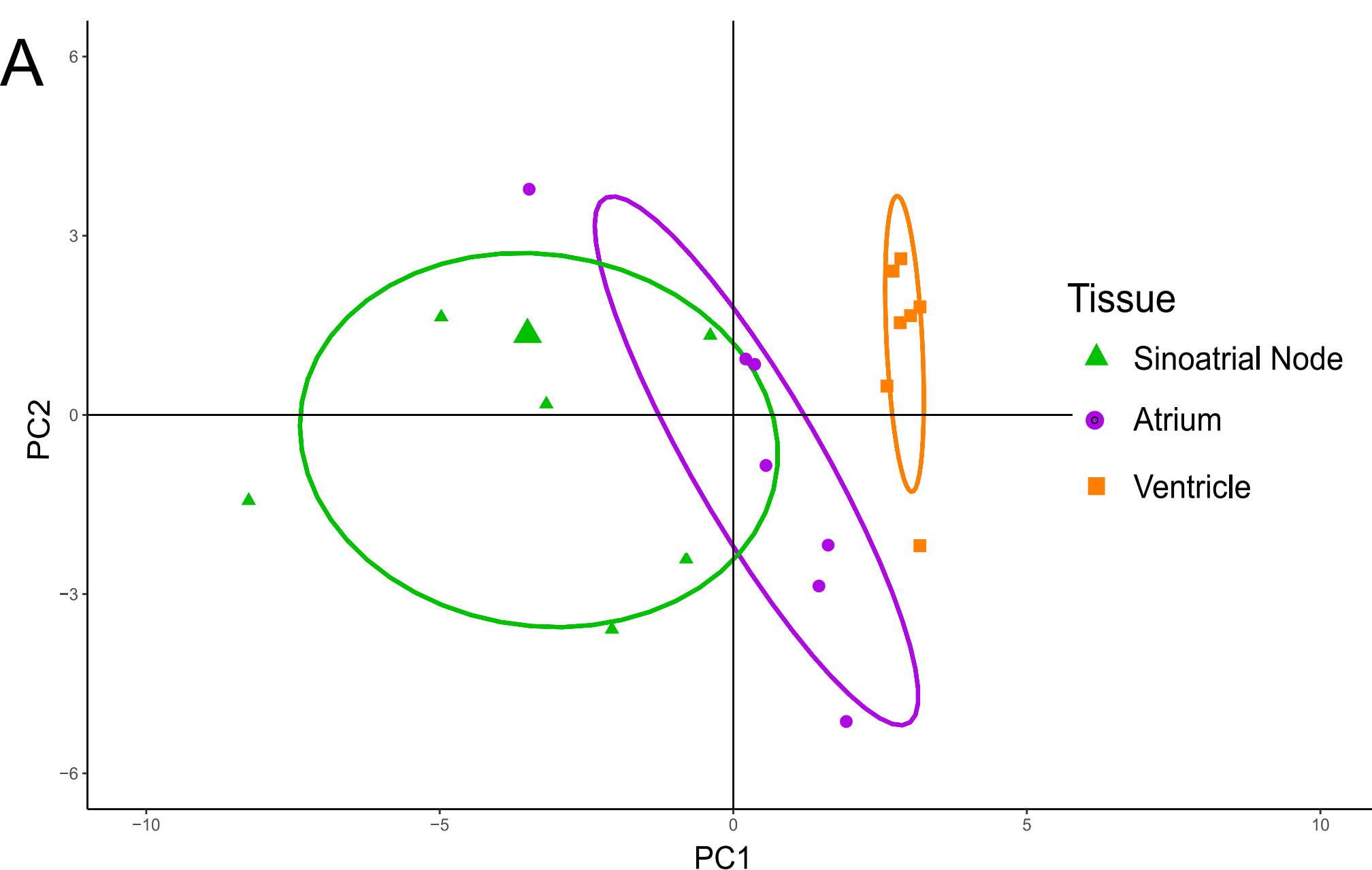
860 **Table 1 - Intrinsic heart rate measurements of fish both with and without a pacemaker intrinsic**
861 **heart rate resetting response, at a variety of temperature acclimations, time points and**
862 **temperatures.**

Fish Group	Temperature Treatment	Saline Measurement Temperature (°C)	Time at Treatment Temperature	Intrinsic Heart Rate	s.e.m.	N
Pacemaker Rate Reset (Group 1)	4°C	4	6 weeks	36.1	0.9	4
	Acute change from 12°C to 4°C	4	0 h (also >3 weeks (3-4 weeks) acclimation to 12°C measured at 4°C)	33.4	0.7	6
			1 h	31.8	0.9	4
			8 h	37.7	1.2	7
			24 h	37.7	1.2	6
	Acute change from 4°C to 12°C	12	0 h (also >3 weeks (4-6 weeks) acclimation to 4°C measured at 12°C)	56.8	1.2	8
			1 h	50.8	1.5	7
			8 h	52.0	1.0	10
			24 h	50.3	1.5	6
	4°C to 4°C	12	1 h	57.9	1.9	8

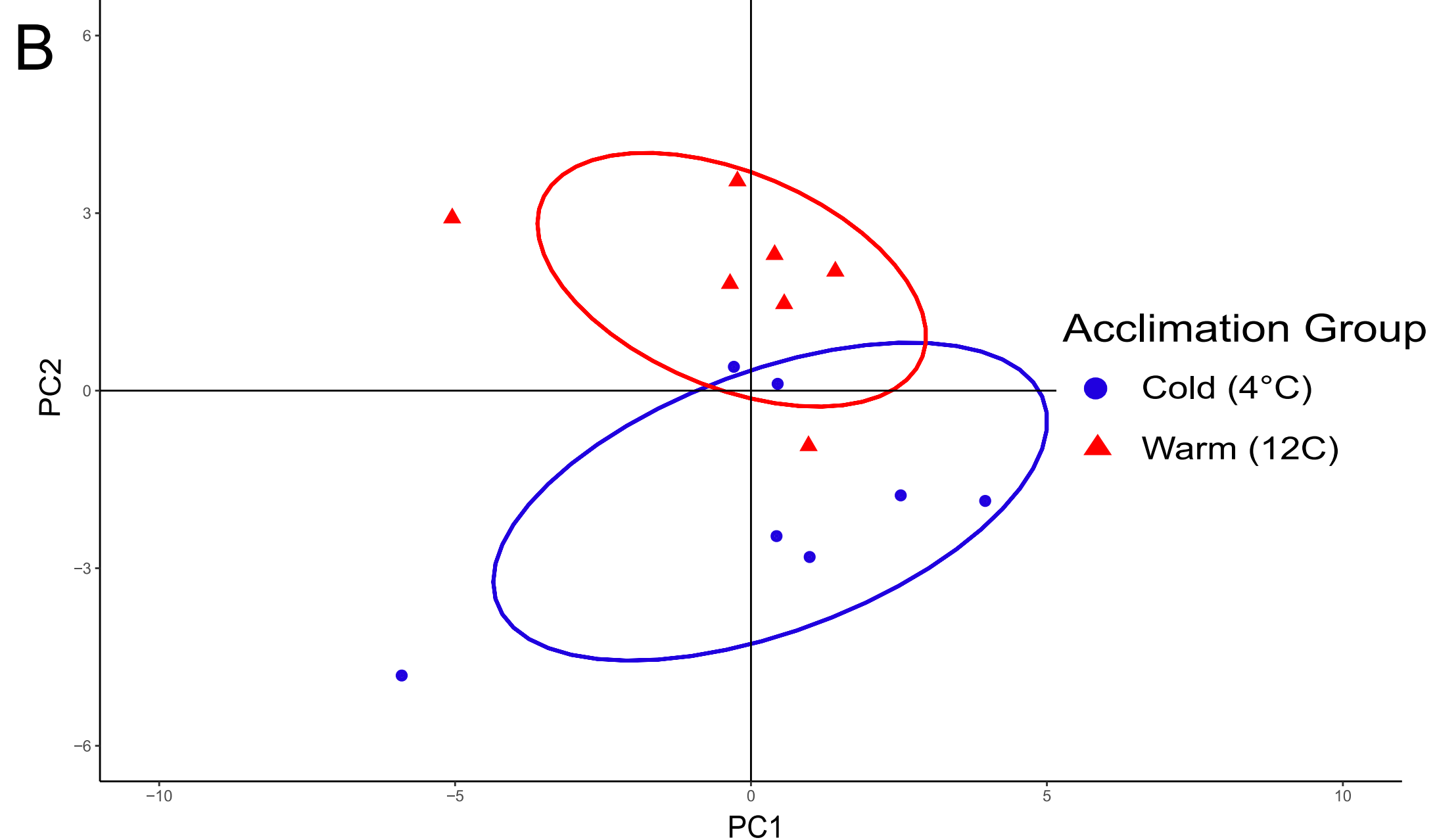
Pacemaker Rate not Reset (Group 2)	Acute change from 12°C to 4°C	4	0 h	30.6	1.7	4
			24 h	29.2	0.6	8
	Acute change from 4°C to 12°C	12	0 h	52.7	2.2	5
			24 h	57.5	2.7	8
	4°C	4	10-12 weeks	29.9	1.1	7
	12°C	4	10-12 weeks	29.4	1.1	7
	4°C	12	10-12 weeks	53.3	2.8	8
	12°C	12	10-12 weeks	52.5	2.3	8

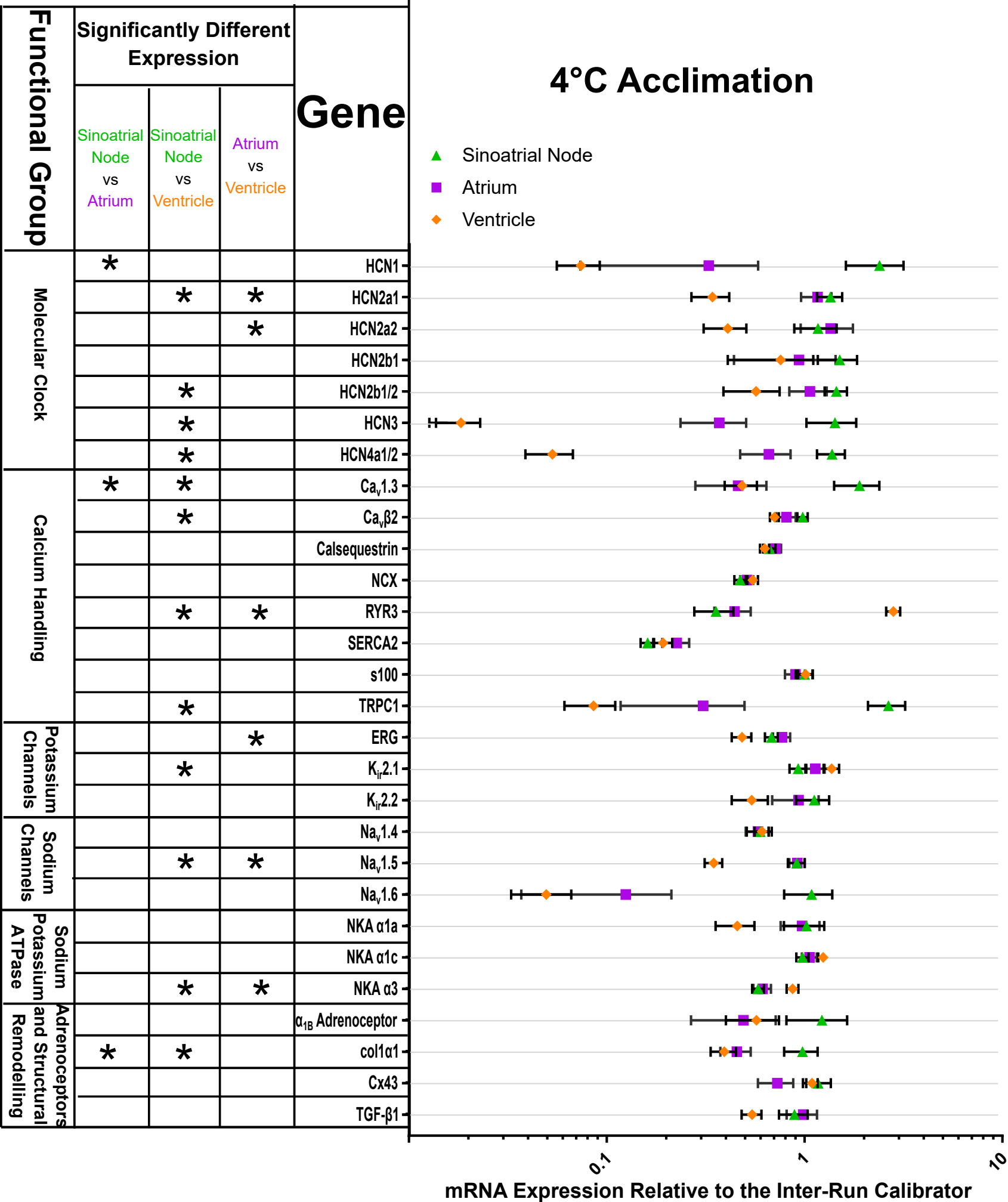


A



B

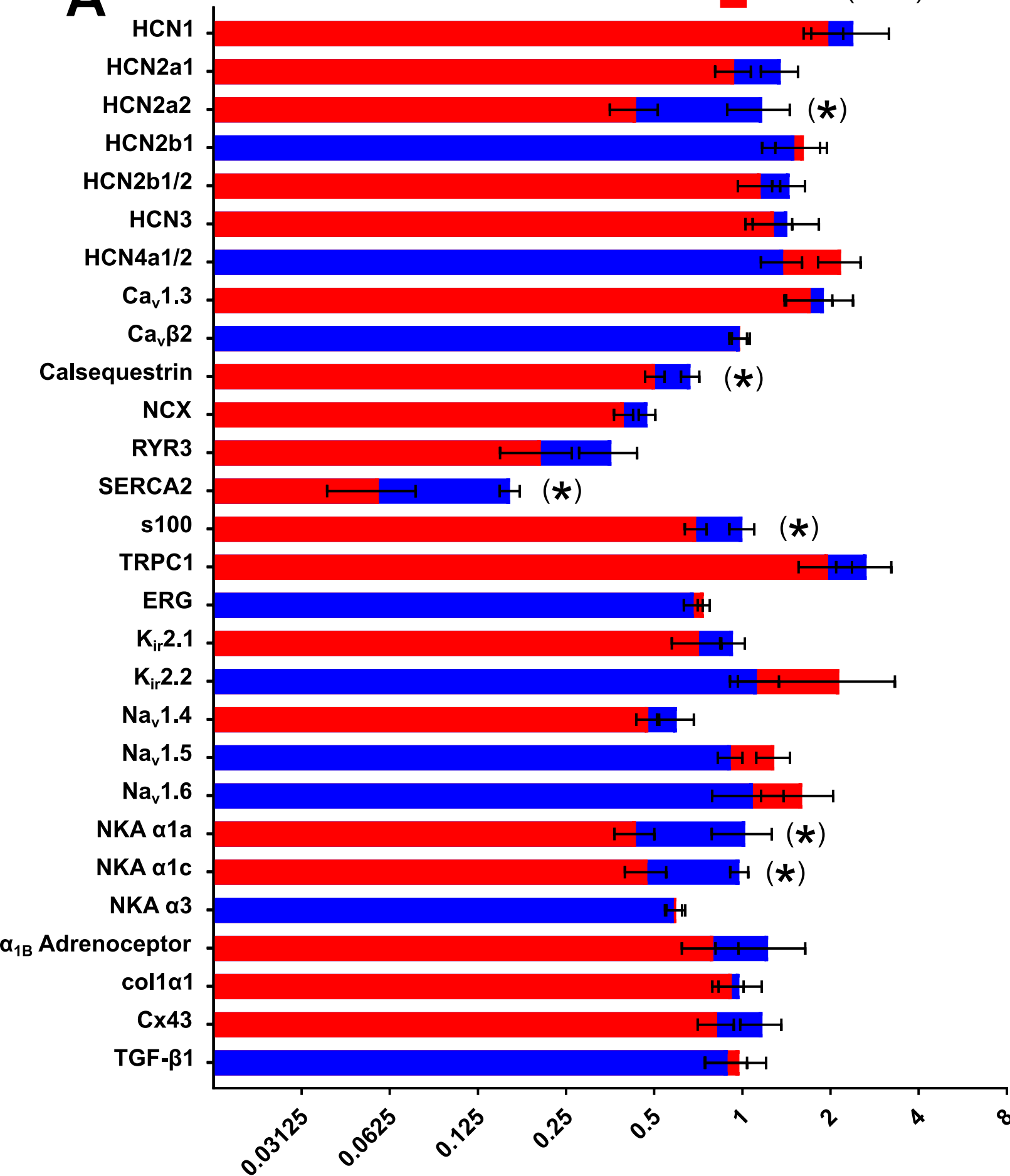




Sinoatrial Node

A

Cold (4°C) Acclimated
Warm (12°C) Acclimated

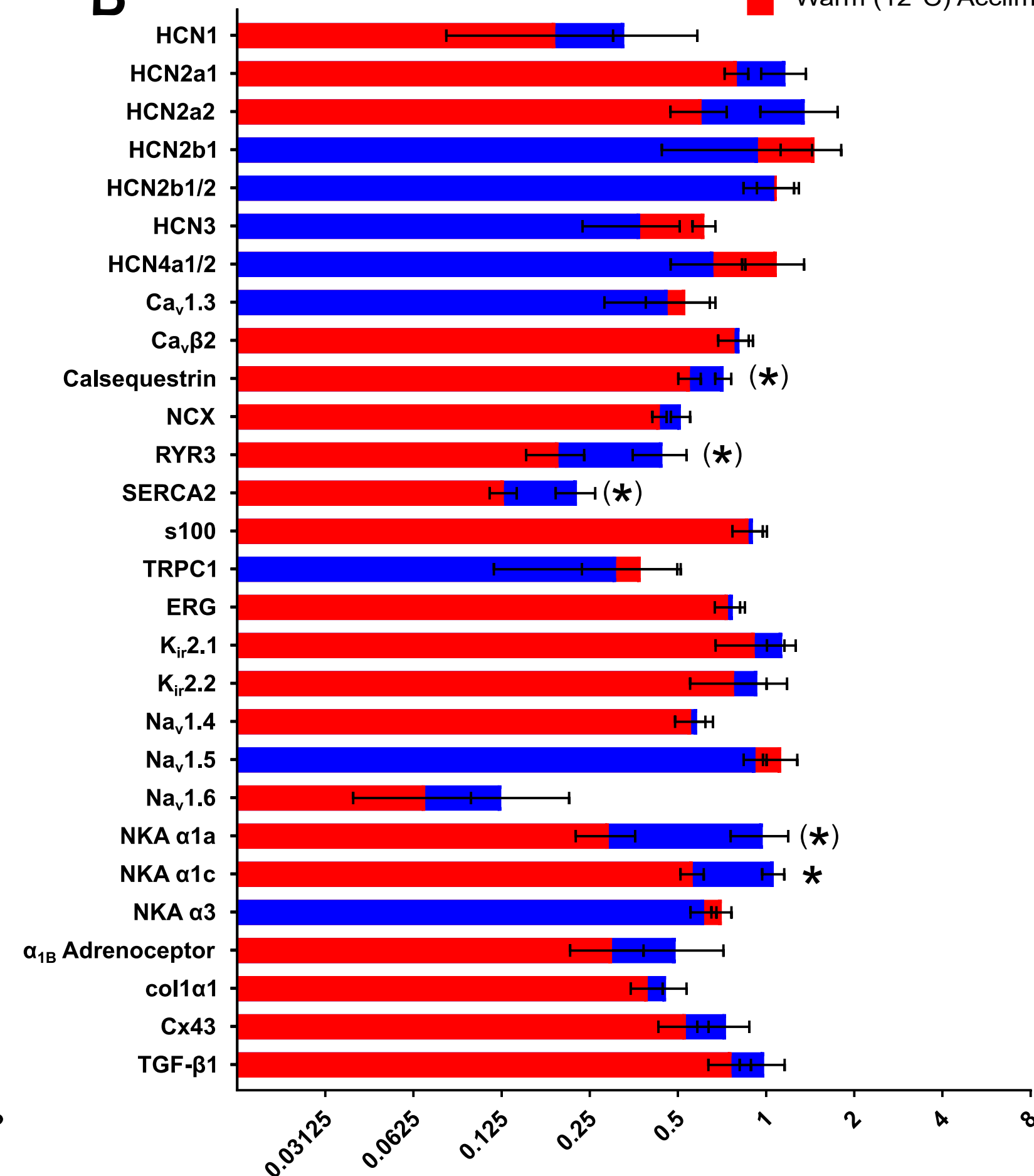


mRNA Expression Relative to Inter-Run Calibrator

Atrium

B

Cold (4°C) Acclimated
Warm (12°C) Acclimated

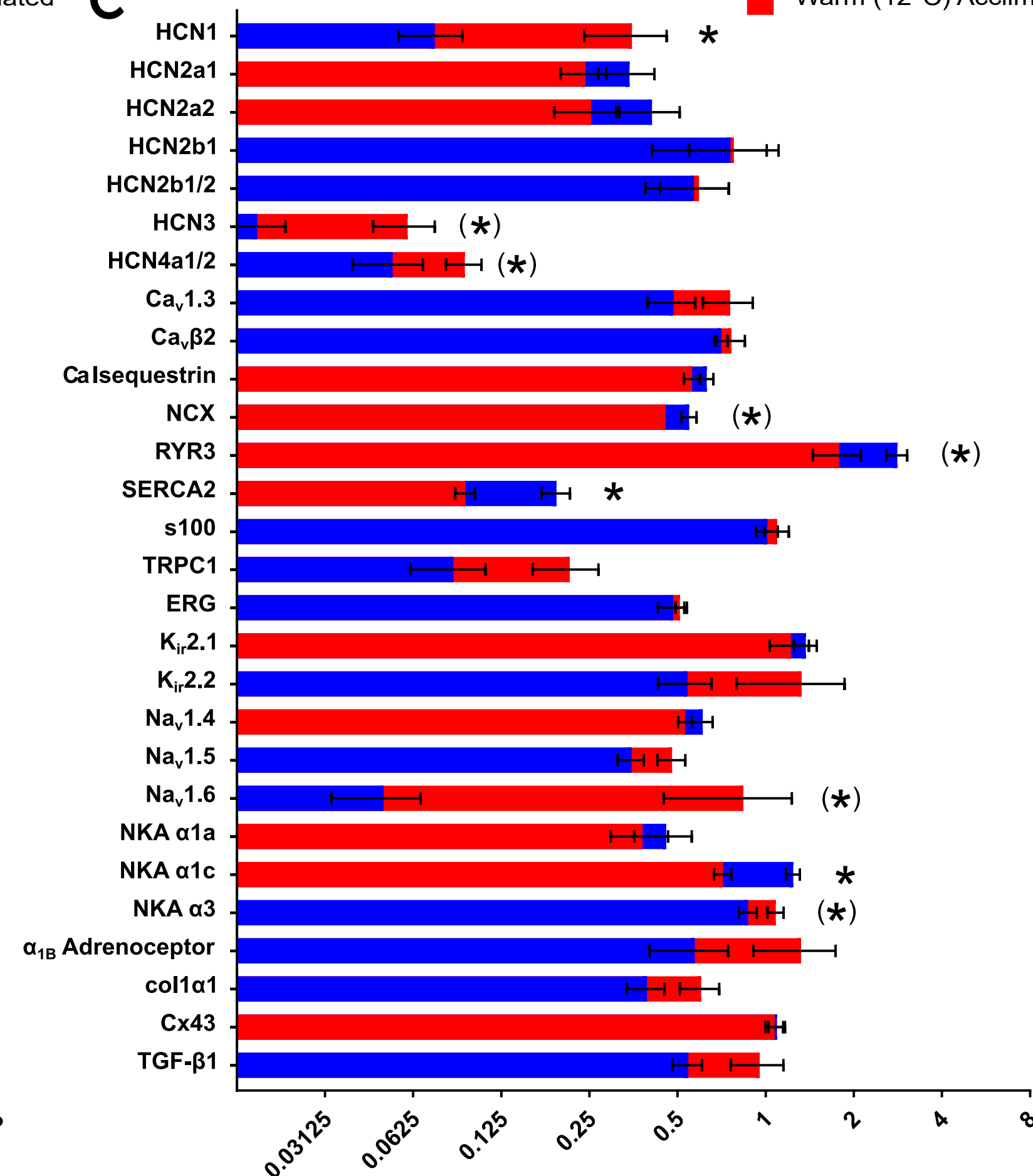


mRNA Expression Relative to Inter-Run Calibrator

Ventricle

C

Cold (4°C) Acclimated
Warm (12°C) Acclimated

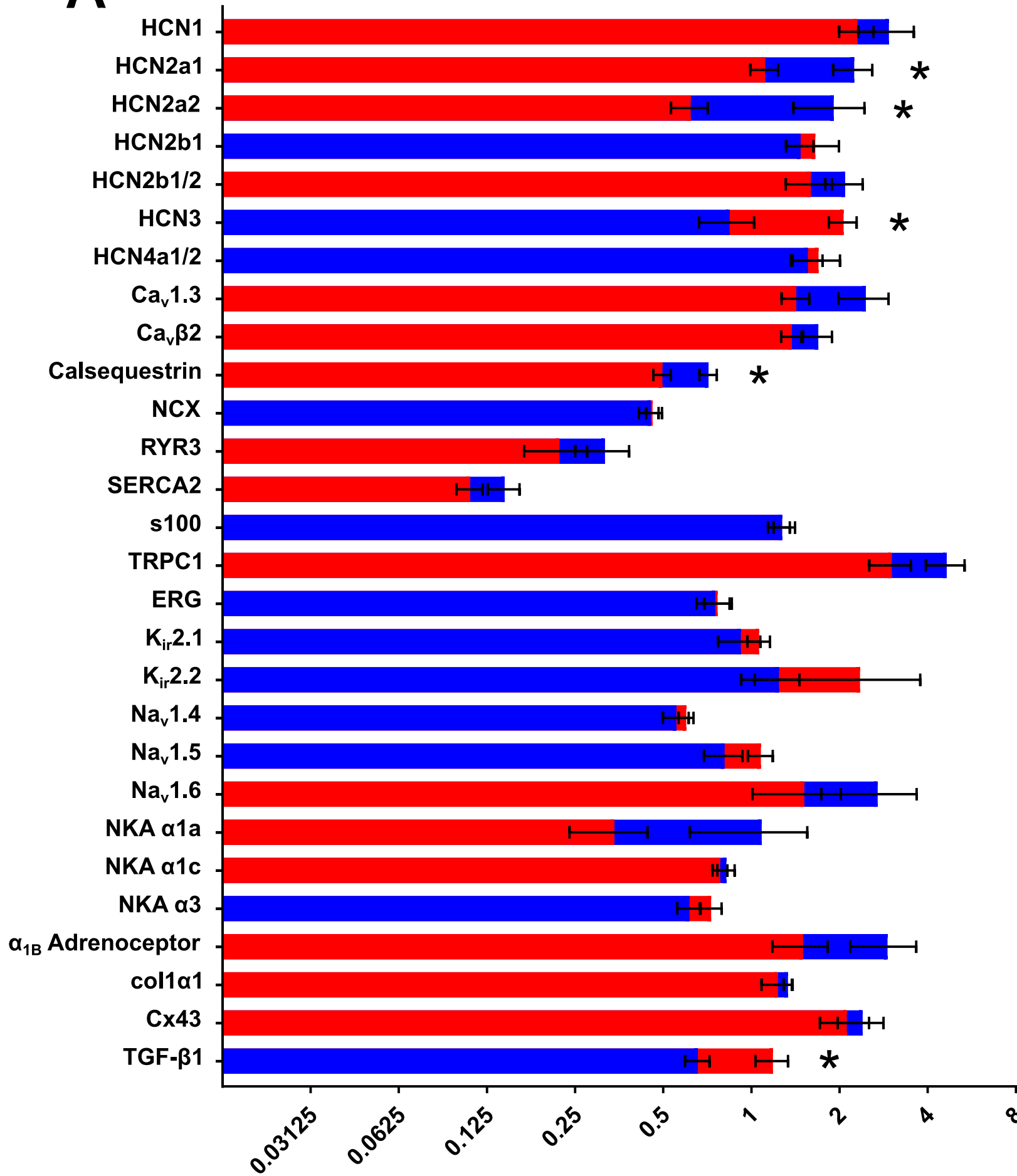


mRNA Expression Relative to Inter-Run Calibrator

Sinoatrial Node

A

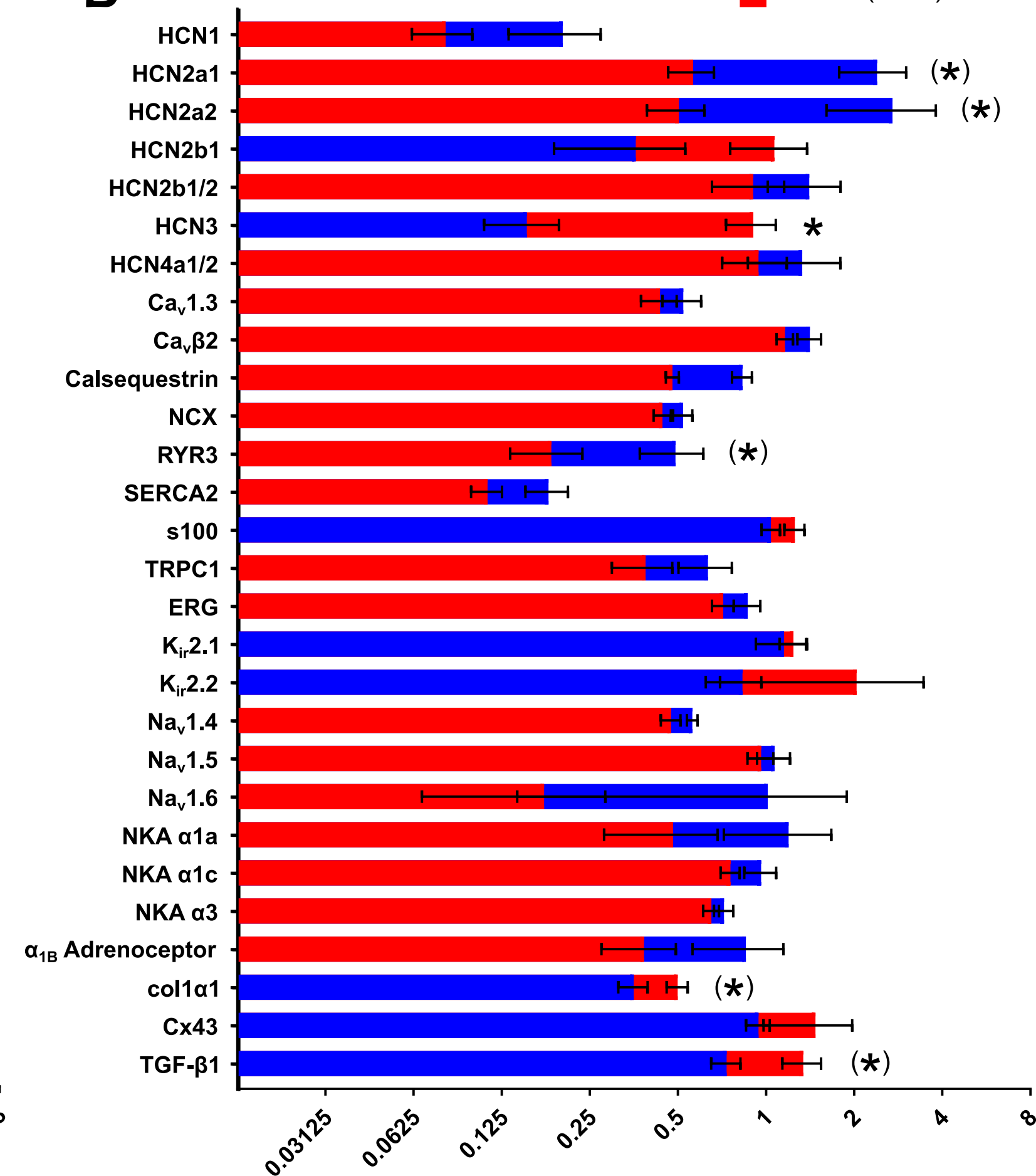
Cold (4°C) Acclimated
Warm (12°C) Acclimated



Atrium

B

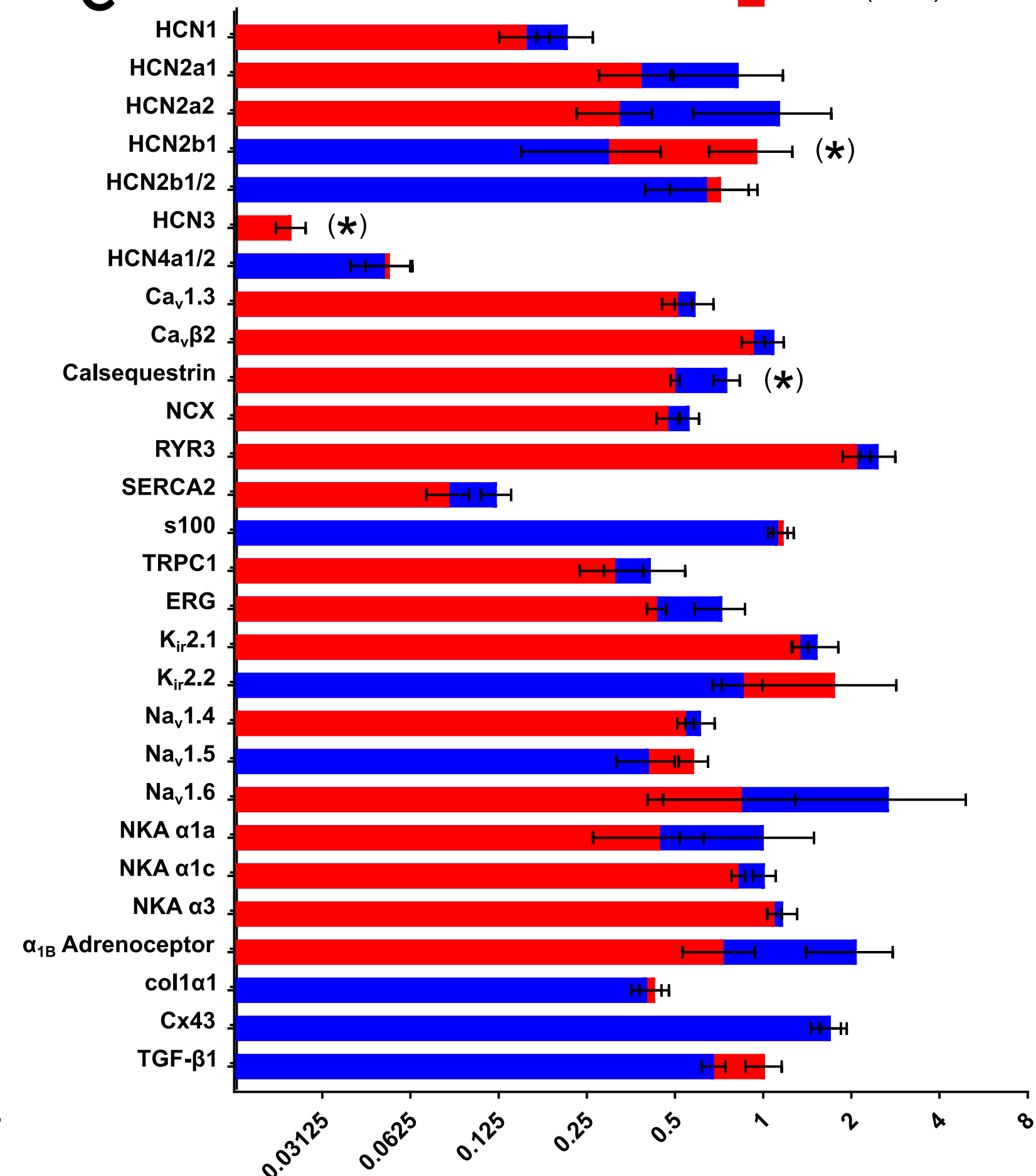
Cold (4°C) Acclimated
Warm (12°C) Acclimated

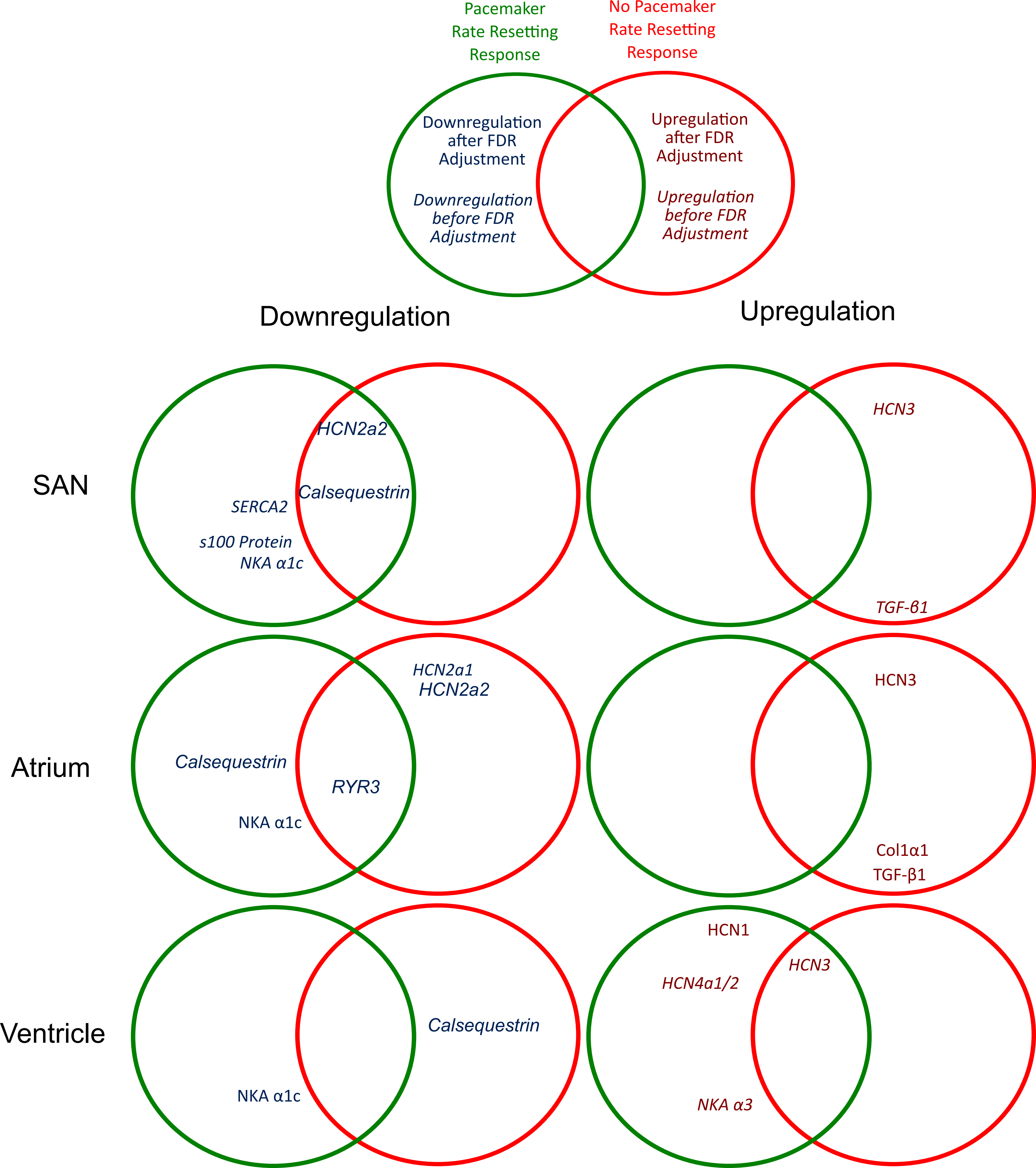


Ventricle

C

Cold (4°C) Acclimated
Warm (12°C) Acclimated

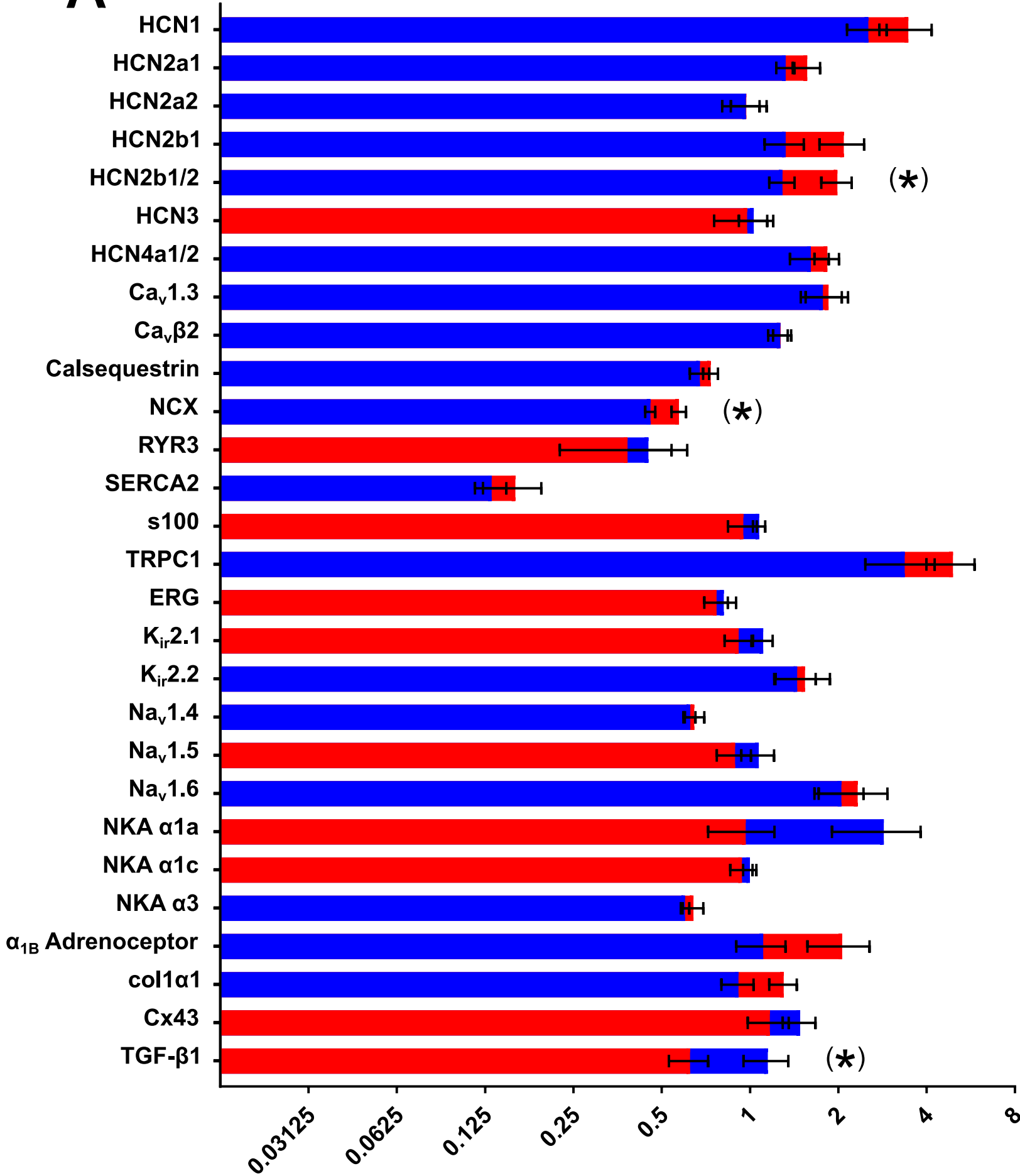




Sinoatrial Node

A

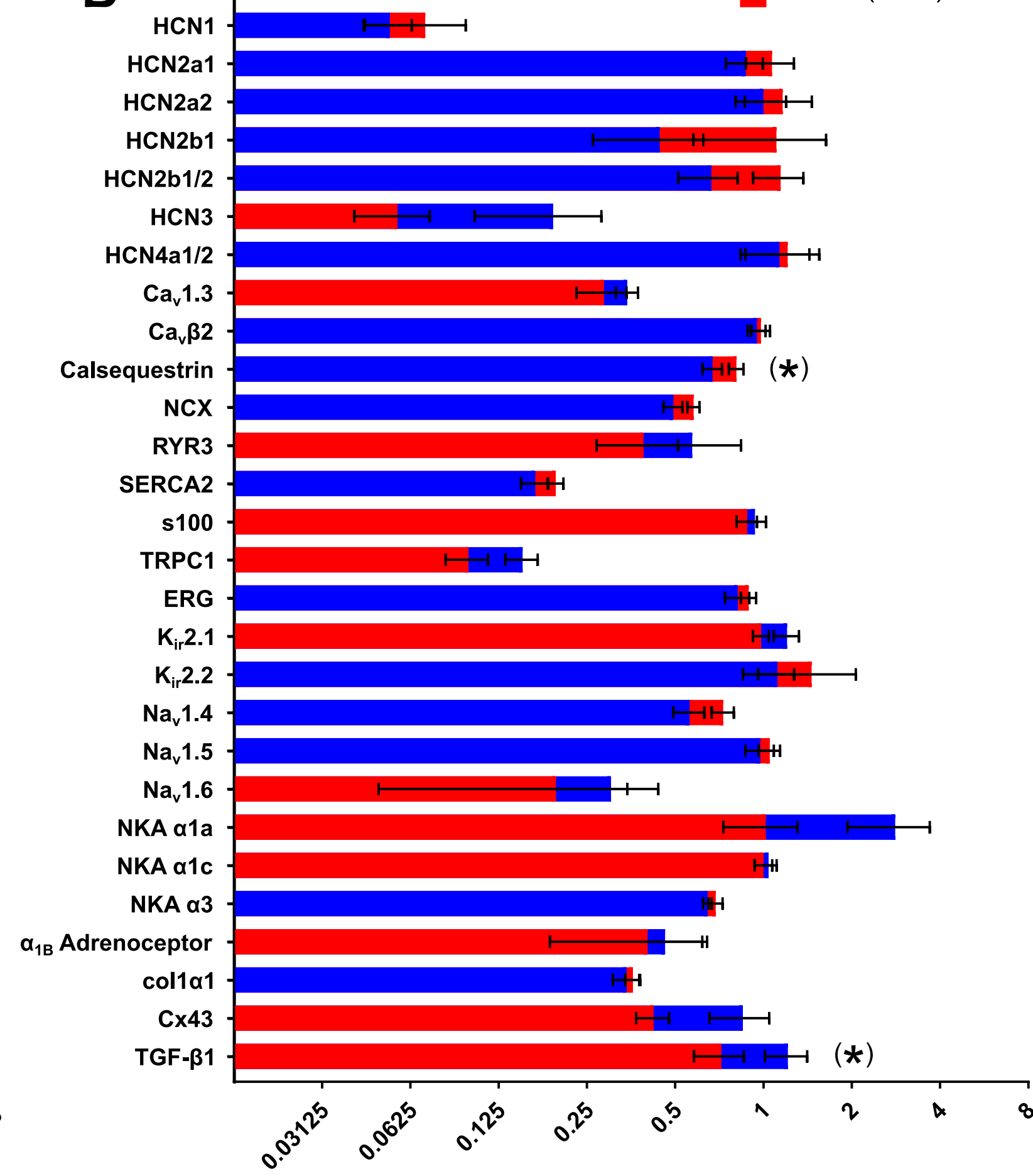
■ Cold (4°C) Acclimated
■ Warm (12°C) Acclimated



Atrium

B

■ Cold (4°C) Acclimated
■ Warm (12°C) Acclimated



Ventricle

C

■ Cold (4°C) Acclimated
■ Warm (12°C) Acclimated

